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The main objective of this project is to evaluate the effects of soy phytoestrogens on reproductive hormones and prostate tissue markers of cell proliferation and androgen action in men at high risk of prostate cancer. The hypothesis is that alteration of endogenous hormones is a mechanism by which soy phytoestrogens prevent prostate cancer. A randomized parallel arm study is being performed, in which 58 men at high risk of prostate cancer were randomized to receive one of three dietary supplements for six months: 1) soy protein isolate containing isoflavones; 2) isoflavone-poor soy powder; or 3) isoflavone-free milk powder. Urine and blood is collected at 0, 3 and 6 mo, for evaluation of serum hormones and prostate specific antigen, as well as urinary estrogen and phytoestrogen metabolites. At 0 and 12 mo, prostate biopsies aperformed to evaluate prostate tissue expression of apoptosis (Bax, Bcl-2), proliferation (PCNA), and androgen receptor density. We found isoflavone-rich soy protein isolate suppressed androgen receptor density, increased urinary estrogen excretion and increased the 2:16-OH estrone ratio in the urine. We also observed a trend toward a lower rate of prostate cancer in the men in the soy groups compared to the men in the milk group

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INTRODUCTION

The low risk of prostate cancer in Asia is thought to be due to dietary factors, including soy consumption. Studies showing an inverse association between prostate cancer risk and urinary excretion of soy phytoestrogens suggest that phytoestrogens contribute to the cancer-preventive effects of soy. One mechanism by which soy phytoestrogens are thought to be cancer-preventive is *via* reduction of endogenous sex hormones known to stimulate prostate cell growth. Despite the interest in soy phytoestrogens for prevention of prostate cancer, there have been no studies in men to evaluate the effects of soy phytoestrogen consumption on sex steroids and prostate tissue biomarkers, and no studies evaluating effects of phytoestrogen metabolism on sex steroids in men.

The main objective of this project is to evaluate the effects of soy phytoestrogen consumption on reproductive hormones and prostate tissue markers of cell proliferation and androgen action in men at high risk of prostate cancer. The underlying hypothesis is that alteration of endogenous hormones is a mechanism by which soy phytoestrogens prevent prostate cancer.

The specific aims of this study (SoyCaP) are to compare the effects of consumption of phytoestrogen-containing soy protein, phytoestrogen-free soy protein, and milk protein, on risk factors for prostate cancer (endogenous hormones, prostate specific antigen, prostate tissue markers of cell proliferation and hormone action), in men at high risk for prostate cancer. Comparing the three groups will enable us to distinguish the specific effects of soy phytoestrogens from effects caused by other soy components. A randomized parallel arm study will be performed, in which 63 men at high risk of prostate cancer will be randomized to receive one of three dietary supplements for six months: 1) soy powder containing 1 mg phytoestrogens/kg body weight; 2) phytoestrogen-free soy powder; and 3) phytoestrogen-free milk powder. Urine and blood will be collected at 0, 3 and 6 months, for evaluation of serum hormones (testosterone, dihydrotestosterone, androstenedione, dehydroepiandrosterone, estradiol, estrone, 3α, 17β-androstanediol glucuronide, sex hormone binding globulin) and prostate specific antigen, as well as urinary estrogen and phytoestrogen metabolites. Before and after the intervention, prostate biopsies will be performed to evaluate prostate tissue expression of apoptosis (Bax and Bcl-2), proliferation (proliferating cell nuclear antigen (PCNA)), epidermal growth factor receptor (EGFr), estrogen receptor beta (ERβ) and androgen receptor (AR)density.

Data from *in vitro*, animal and epidemiological studies suggest that androgens and estrogens play a role in prostate carcinogenesis. Soy isoflavones have been shown to alter sex steroids in women in a potentially beneficial direction, yet such studies in men have not been reported. Studies of the hormonal effects of soy isoflavones in men will contribute to our knowledge of the cancer-preventive mechanisms of soy isoflavones, and may lead to dietary recommendations for prevention of prostate cancer.

BODY

According to the original statement of work, the following tasks were to be performed during the two years of this project:

Task 1: Work with IRB and approval from Army's Office of Research Protection and coordinate with Veteran's Administration to establish all study protocols (months 0-6).

Task 2: Determine the effects of soy phytoestrogen consumption on serum hormones, sex hormone binding globulin (SHBG) and prostate specific antigen (PSA); urinary phytoestrogens and estrogen metabolites; and prostate biopsy biomarkers in men at high risk of prostate cancer.

- Analyze samples from cohort #1 (30 men): serum hormones and SHBG by RIA; serum free and total PSA by ELISA; urine estrogen metabolites and phytoestrogens by GC-MS; biopsy slides by immunohistochemistry (months 6-7)

- Recruit 15 men at high risk of prostate cancer (cohort #2) and randomize into three intervention groups (month 9-12)
- Perform feeding study in cohort #2; process and store serum, urine and biopsy slides (months 9-12)
- Analyze samples from cohort #2: serum hormones and SHBG by RIA; serum free and total PSA by ELISA; urine estrogen metabolites and phytoestrogens by GC-MS; biopsy slides by immunohistochemistry (months 9-12)

- Recruit 15 men at high risk of prostate cancer (cohort #3) and randomize into three intervention groups (month 9-12)
- Perform feeding study in cohort #3; process and store serum, urine and biopsy slides (months 9-12)
- Analyze samples from cohort #3: serum hormones and SHBG by RIA; serum free and total PSA by ELISA; urine estrogen metabolites and phytoestrogens by GC-MS; biopsy slides by immunohistochemistry (months 9-12)

Task 3: Perform data analyses and prepare manuscripts for publication (months 13-24)

All tasks have been completed. The final year was spent on preparing manuscripts for publication, presenting the data at professional meetings, and completing the requirements for a doctoral degree. Three manuscripts were prepared for publication and all have now been published. The data are summarized in the results section of this report and the peer-reviewed publications have been appended.

Recruitment Summary

The last study year: October 2005 - December 2006:

From October 2005 - December 2006

To date a total of 90 subjects have been enrolled, out of which 56 have completed the study, 10 have dropped out, and 24 consented but never started the study. Data from 2 subjects that completed 3 months of the study with good compliance were analyzed and included in results.

Table 1. Enrollment Summary

	Completed 6 months	Currently completing study	Withdrew after starting	Consented but did not start	Total enrollment
Prior to grant	37	0	9	19	65
10/05–12/06	19	0	1	5	25
TOTAL	56	0	10	24	90

Of the subjects enrolled, 5 individuals (18.5%) did not start the study as a result of inconvenience or placement on a physician monitored weight-loss plan.

One person withdrew after the starting the study. The reason for withdrawal was discomfort with the powder i.e. feeling of being bloated.

Results

Dietary Data and Anthropometrics: No differences were observed in baseline anthropometrics, cancer status and dietary intake (Table 1), except that the SPI (-) group had higher baseline intake of protein, zinc and calcium, and the MPI group had a higher baseline body weight (Table 2).

Dietary intake of protein calcium and vitamin-D intake increased in all groups and were significantly higher than baseline values. Additionally, fat intake was reduced in the SPI (-) group at 3 months (Table 2). However, these dietary and anthropometric differences between groups were unrelated to changes in serum hormone concentrations and prostatic steroid-receptor expression profiles.

TABLE 1: Baseline characteristics of subjects [1]

	SPI (+)	SPI (-)	MPI
	n = 20	n = 20	n = 18
Age (y)	68 ± 8	68 ± 5	68 ± 7
Body wt (kg)	91 ± 16 ab	88 ± 12 a	98 ± 15 b
Height (cm)	175 ± 7	173 ± 8	176 ± 8
BMI (kg/m^2)	30 ± 5	29± 4	32 ± 6
Prostate Cancer Markers [2]			
PIN (n (%))	18 (90)	18 (90)	14 (78)
ASAP (n (%))	3 (15)	7 (35)	4 (22)
CaP (n (%))	2 (10)	1 (5)	2 (12)

[1] All values are means ± SD except prostate cancer markers which are n (%).
[2] Prostate cancer markers PIN, ASAP, and CaP are not mutually exclusive.
[ab] Means in a row without a common letter differ ($p < 0.05$).

TABLE 2: Anthropometrics and dietary intake [1]

	SPI+ n = 20 [2]	SPI- n = 20	MPI n = 18
Weight (kg)			
Baseline	91 ± 16 ab	88 ± 12 a	98 ± 15 b
3 Mo	91 ± 16 ab	87 ± 12 a	98 ± 15 b
6 Mo	90 ± 16 ab	87 ± 13 a	99 ± 15 b
Height (cm)			
Baseline	175 ± 16	173 ± 8	176 ± 8
BMI (kg/m^2)			
Baseline	30 ± 5	29 ± 4	32 ± 6
3 Mo	30 ± 5	29 ± 4	32 ± 6
6 Mo	30 ± 5	29 ± 4	32 ± 6
Energy Intake (kcal/d) [3]			
Baseline	2140 ± 620	2260 ± 660	2070 ± 520
3 Mo	2220 ± 720	2030 ± 390	2180 ± 510
6 Mo	2240 ± 410	2120 ± 670	2330 ± 410
Protein (g /d)			
Baseline	83 ± 21 a	100 ± 24 b	81 ± 25 a
3 Mo	* 118 ± 24	* 117 ± 16	* 121 ± 30
6 Mo	* 118 ± 21	* 124 ± 29	* 120 ± 18
Carbohydrate (g/d)			
Baseline	256 ± 106	262 ± 118	236 ± 59

3 Mo	246 ± 97	230 ± 82	232 ± 75
6 Mo	251 ± 61	232 ± 89	256 ± 68
Total Fat (g/d)			
Baseline	86 ± 33	93 ± 32	88 ± 24
3 Mo	80 ± 39	* 74 ± 18	73 ± 30
6 Mo	83 ± 34	80 ± 34	89 ± 26
Saturated Fat (g/d)			
Baseline	27 ± 11	34 ± 14	28 ± 11
3 Mo	27 ± 13	* 26 ± 7	24 ± 12
6 Mo	26 ± 10	29 ± 14	30 ± 10
Cholesterol (mg/d)			
Baseline	324 ± 202	382 ± 153	301 ± 163
3 Mo	307 ± 131	296 ± 115	312 ± 233
6 Mo	328 ± 147	348 ± 175	329 ± 234
Fiber (g/d)			
Baseline	17 ± 9	18 ± 7	16 ± 5
3 Mo	16 ± 8	17 ± 8	15 ± 7
6 Mo	15 ± 9	16 ± 9	15 ± 5
Vitamin D (μg/d)			
Baseline	4 ± 3	4 ± 5	4 ± 3
3 Mo	* 9 ± 4	* 8 ± 3	* 8 ± 2
6 Mo	* 8 ± 2	* 8 ± 3	* 9 ± 2

Vitamin E (mg/d)			
Baseline	8 ± 7	8 ± 5	6 ± 4
3 Mo	6 ± 4	7 ± 10	6 ± 3
6 Mo	7 ± 7	6 ± 3	6 ± 3
Calcium (mg/d)			
Baseline	890 ± 400 ab	1230 ± 970 b	760 ± 360 a
3 Mo	* 2260 ± 440	* 2120 ± 350	* 2200 ± 380
6 Mo	* 2180 ± 290	* 2340 ± 840	* 2190 ± 340
Selenium (mg/d)			
Baseline	0.08 ± 0.05	0.09 ± 0.03	0.08 ± 0.05
3 Mo	0.08 ± 0.03	* 0.06 ± 0.03	0.10 ± 0.11
6 Mo	0.07 ± 0.03	* 0.07 ± 0.02	0.44 ± 1.6
Zinc (mg/d)			
Baseline	10 ± 6 a	14 ± 5 b	10 ± 5 a
3 Mo	11 ± 4	10 ± 8	10 ± 3
6 Mo	9 ± 3	10 ± 5	9 ± 3

[1] All values are means ± SD.
[2] Sample sizes listed at column headings are for all time points except the following: 3 mo, MPI (n = 17), and 6 mo, SPI+ (n = 18) and SPI- (n = 18).
[3] 1 kcal = 4.184 kJ
[ab] Means in a row without a common letter differ (P < 0.05).
*Significant within-group change from baseline (P < 0.05).

Serum hormones, SHBG and receptor expression*:* Baseline serum hormone and SHBG concentrations and prostatic steroid-hormone expression levels did not differ between the groups (Tables 3 & 4). Six-month prostatic androgen receptor expression was lower in the SPI (+) group as compared to the MPI group (*P*=0.04) and tended to be lower in the SPI (-) group as compared to the milk group (*P*= 0.09). No differences were observed in estrogen receptor-beta expression (Table 3). Serum concentrations of estradiol, estrone, androstenedione and DHT increased during the intervention in the SPI (-) group, and at 3-months serum estrone and androstenedione concentrations were significantly higher in the SPI (-) group. These differences persisted at 6-months Also higher concentrations of estradiol, and DHEAS were observed at 6-months in the SPI (-) group (Table 4). Serum SHBG concentrations decreased from baseline in all 3 groups and no group differences were observed (Table 4).

TABLE 3: Steroid receptor expression (HSCORE) [1]

	SPI+	SPI-	MPI
Androgen Receptor (AR)			
Baseline	1.37 ± 0.06	1.28 ± 0.06	1.23 ± 0.06
6 Mo	1.26 ± 0.05 *a*	1.30 ± 0.05 *ab*	* 1.42 ± 0.05 *b*
Estrogen Receptor β (ERβ)			
Baseline	1.22 ± 0.06	1.32 ± 0.06	1.23 ± 0.06
6 Mo	1.16 ± 0.06	1.18 ± 0.06	1.26 ± 0.05

[1] Baseline data are unadjusted means ± SEM. All other data are least-squares means adjusted for baseline measurement ± SEM. The number of patients evaluated for AR expression was 14 for SPI+, 16 for SPI-, and 14 for MPI. The number of patients evaluated for ERβ expression was 14 for SPI+, 14 for SPI-, and 15 for MPI.
[ab] Means in a row without a common letter differ (*P* < 0.05).
*Significant within-group change from baseline (*P* < 0.05).

TABLE 4: Serum hormones and SHBG [1]

	SPI+ *n* = 20 [2]	SPI- *n* = 20	MPI *n* = 18
Estradiol (pmol/L)			
Baseline	67 ± 4	66 ± 4	69 ± 3
3 Mo	75 ± 5	* 76 ± 5	* 62 ± 6
6 Mo	69 ± 3 *a*	* 79 ± 3 *b*	66 ± 3 *a*
Estrone (pmol/L)			
Baseline	157 ± 15	141 ± 10	158 ± 8
3 Mo	150 ± 8 *ab*	* 170 ± 8 *b*	146 ± 8 *a*
6 Mo	152 ± 10	* 171 ± 10	150 ± 10
Androstenedione (nmol/L)			
Baseline	2.9 ± 0.3	2.9 ± 0.3	2.5 ± 0.2
3 Mo	3.0 ± 0.2 *a*	3.0 ± 0.2 *ab*	2.8 ± 0.2 *b*
6 Mo	2.6 ± 0.2 *a*	* 3.4 ± 0.2 *b*	2.9 ± 0.2 *ab*
Androstanediol Glucuronide (nmol/L)			
Baseline	19 ± 3	18 ± 5	16 ± 2
3 Mo	17 ± 2 *a*	24 ± 2 *b*	17 ± 2 *a*
6 Mo	16 ± 2	20 ± 2	18 ± 2
DHEAS (nmol/L) [†]			
Baseline	2202 ± 390	2052 ± 300	1977 ± 370
3 Mo	2040 ± 103 *a*	2715 ± 103 *b*	2126 ± 103 *a*

6 Mo	1937± 154 *a*	2372 ± 146 *b*	1946 ± 150 *a*
DHT (pmol/L)			
Baseline	1547 ± 190	1354 ± 170	1072 ± 110
3 Mo	1242 ± 81	* 1076 ± 79	1119 ± 100
6 Mo	1215 ± 94	1174 ± 89	1229 ± 105
Testosterone (nmol/L)			
Baseline	12 ± 1	13 ± 1	12 ± 1
3 Mo	13 ± 0.5	13 ± 0.6	11 ± 0.6
6 Mo	13 ± 0.6	13 ± 0.5	12 ± 0.6
Free Testosterone (pmol/L)			
Baseline	33 ± 3	34 ± 2	29 ± 2
3 Mo	33 ± 1	33 ± 1	32 ± 1
6 Mo	32 ± 1	32 ± 1	31 ± 1
SHBG (nmol/L) [‡]			
Baseline	63 ± 7	64 ± 8	69 ± 9
3 Mo	* 56 ± 3	* 56 ± 2	* 56 ± 3
6 Mo	* 54 ± 3	* 61 ± 3	* 58 ± 3

[1] Baseline data are unadjusted means ± SEM. All other data are least-squares means adjusted for baseline measurement ± SEM, except androstenedione which is additionally adjusted for interaction between treatment and baseline.
[2] Sample sizes listed at column headings are for all time points except: 3 mo MPI (*n* = 17), and 6 mo SPI+ (*n* = 18) and SPI- (*n* = 19).
[†] Sample sizes differed from other hormones due to excluded data. At 3 mo, SPI+ (*n* = 19) and SPI- (*n* = 19). At 6 mo, SPI+ (*n* = 17) and SPI- (*n* = 19).
[‡] Sample sizes differed from other hormones due to excluded data. At 3 mo, SPI+ (*n* = 19), and at 6 mo, SPI+ (*n* = 18).
[ab] Means in a row without a common letter differ ($P < 0.05$).
*Significant within-group change from baseline ($P < 0.05$).

Urinary Estrogen Metabolites: At baseline, urinary estrogen metabolites did not differ between the 3-groups with the exception of 2-methoxyestradiol which was significantly higher in the SPI (+) group as compared to the MPI group. At 3-months urinary estradiol and was significantly higher while 16α-hydroxyestrone tended to be higher in SPI (+) and SPI (-) groups as compared to the MPI group. These differences in urinary estradiol concentrations persisted at 6-months. Higher urinary 2-hydroxyestradiol levels were also observed in the soy groups as compared to the MPI group. The 6-month 2:16-hydroxyestrone ratio (2-hydroxyestrone/16α-hydroxyestrone) tended to be higher in the SPI (+) group as compared to the MPI group (Table 5).

TABLE 5: Urinary estrogen metabolites (nmol/d) in men at high risk of prostate cancer that consumed various protein isolates for 6 mo[1]

	SPI (+) *n* = 19 [2]	**SPI (-)** *n* = 19 [2]	**MPI** *n* = 17 [2]
Estradiol (E2)			
Baseline	51 (34, 74)	42 (27, 63)	49 (29, 81)
3 mo	94 (65, 135) [a]*	76 (53, 111) [a]*	44 (30, 66) [b]
6 mo	91 (63, 132) [a]*	90 (63, 129) [a]*	50 (34, 72) [b]
Estrone (E1)			
Baseline	20 (15, 28)	18 (13, 26)	25 (20, 31)
3 mo	26 (20, 34)	21 (16, 28)	22 (16, 29)
6 mo	37 (28, 49) [a]*	27 (20, 35) [b]*	23 (17, 30) [b]

2-methoxyestradiol (2-ME2)			
Baseline	57 (39, 84) [a]	42 (31, 56) [ab]	31 (21, 47) [b]
3 mo	39 (26, 58)	42 (28, 62)	37 (24, 57)
6 mo	26 (18, 38)	36 (25, 52)	34 (23, 50)
2-methoxyestrone (2-ME1)			
Baseline	9.1 (7, 12)	9.5 (6, 15)	8.1 (6, 12)
3 mo	7.6 (5, 12)	9.5 (6, 15)	8.9 (6, 14)
6 mo	9.8 (7, 15)	9.2 (6, 14)	7.9 (5, 12)
Estriol (E3)			
Baseline	55 (42, 71)	28 (14, 57)	47 (29, 78)
3 mo	28 (19, 42) [a]	56 (38, 84) [b]	41 (27, 63) [ab]
6 mo	31 (19, 49)	45 (28, 72)	42 (26, 67)
2-hydroxyestradiol (2-OH-E2)			
Baseline	7.3 (5, 12)	4.8 (3, 8)	5.9 (3, 11)
3 mo	7.2 (5, 11)	6.9 (4, 11)	5.6 (4, 9)
6 mo	5.6 (4, 8) [a]	8.3 (6, 12) [a]	* 3.0 (2, 4) [b]
2-hydroxyestrone (2-OH-E1)			
Baseline	20 (13, 30)	21 (13, 33)	26 (19, 36)
3 mo	21 (14, 30)	23 (16, 34)	26 (17, 38)
6 mo	29 (21, 41)	25 (18, 35)	21 (15, 30)

16α-hydroxyestrone (16α-OH-E1)			
Baseline	6.0 (4, 9)	5.7 (4, 9)	6.9 (5, 10)
3 mo	7.4 (5, 11) [a]	7.9 (5, 11) [a]	4.5 (3, 7) [b]
6 mo	6.0 (4, 9)	7.3 (5, 11)	6.6 (5, 10)
2:16 OH-E1 ratio (mean ± SE)			
Baseline	7.8 ± 1	7.5 ± 1	7.8 ± 2
3 mo	8.0 ± 2	8.5 ± 2	10.4 ± 2
6 mo	11.3 ± 2 [a]	8.2 ± 2 [ab]	5.1 ± 2 [b]

[1] Baseline data are unadjusted geometric means (95% CI) except 2:16 OH-E1 ratio data which are means ± SE. All other data are least-squares geometric means adjusted for baseline measurement ± 95% CI, except 16α-OH-E1 which is additionally adjusted for baseline weight and 2:16 OH-E1 ratio data which are least-squares means ± SE and were analyzed on the original scale. Means in a row with superscripts without a common letter differ ($P < 0.05$). *Different from baseline ($p < 0.05$).
[2] Sample sizes listed at column headings are for all time points except the 3 mo MPI ($n = 16$), 6 mo SPI+ ($n = 17$), and 6 mo SPI- ($n = 18$) timepoints.

Prostate Cancer Biomarkers, PSA and prostate volume: No between-group differences in baseline aggregate antigen expression HSCORES, serum total and free PSA concentrations, prostate volume and PSA density (serum PSA/ prostate volume) were observed (Table: 6,7, and 8). Serum total PSA and free PSA and PSA percent was unaltered by the treatments. Although at 6-months, greater prostate volume was observed in the SPI (-) group as compared to the MPI group, PSA density did not differ between groups (Tables 6 and 7).

Prostatic Bax expression was lower in SPI (-) group as compared to MPI group (P = 0.03) and tended to be lower in the SPI (+) group as compared to the MPI group (P =0.10) after 6-months of interventions. PCNA expression was reduced from baseline in the SPI (-) group, however no group differences were observed at 6-months. No changes in prostatic Bcl-2 and EGFr expression or Bax:PCNA /Bax:Bcl-2 ratio were observed (Table 8).

Table 6: Prostate volume and PSA density differences from baseline

	SPI+ n = 10	**SPI-** n = 13	**MPI** n = 15	**P-Value**
Prostate Volume (cm^3)				
Baseline	52 ± 5	47 ± 5	54 ± 6	0.6709
6 Mos Change	-4.3 ± 3 *ab*	1.6 ± 2 *a*	-5.5 ± 2 *b*	0.0951
PSA Density (ng/mL/cc)				
Baseline	0.1 ± 0.03	0.09 ± 0.02	0.1 ± 0.02	0.8255
6 Mos Change	0.0001 ± 0.01	-0.003 ± 0.01	-0.005 ± 0.01	0.9614

Baseline data are unadjusted means ± standard errors. Differences are post-intervention minus baseline and are least-squares means adjusted for baseline measurement ± standard errors. Pre-planned treatment pairwise comparisons are between groups within each row: means that do not share letters are significantly different ($p < 0.05$).

Table 7: Serum PSA differences from baseline

	SPI+ n = 20	SPI- n = 20	MPI n = 18	
Total PSA (ng/mL)				
Baseline	5.4 ± 1	5.0 ± 1	5.1 ± 1	0.9611
3 Mos Change	-0.8 ± 0.5	-0.8 ± 0.5	-0.6 ± 0.6	0.9373
6 Mos Change	-0.5 ± 0.6	-0.8 ± 0.6	-0.2 ± 0.6	0.7880
Free PSA (ng/mL)				
Baseline	0.9 ± 0.09	0.8 ± 0.1	0.9 ± 0.2	0.7259
3 Mos Change	-0.09 ± 0.09	0.04 ± 0.09	-0.10 ± 0.1	0.4867
6 Mos Change	-0.07 ± 0.07	-0.02 ± 0.07	-0.06 ± 0.07	0.8572
PSA Percent				
Baseline	22 ± 2	19 ± 2	22 ± 2	0.5138
3 Mos Change	-0.21 ± 1	0.67 ± 1	-0.74 ± 1	0.6055
6 Mos Change	1.03 ± 1	1.18 ± 1	-0.22 ± 1	0.7196

Baseline data are unadjusted means ± standard errors. Differences are post-intervention minus baseline and are least-squares means adjusted for baseline measurement ± standard errors. Pre-planned treatment pairwise comparisons are between groups within each row: means that do not share letters are significantly different ($p < 0.05$).
Sample sizes listed at column headings are at baseline. At 3 mos, MPI: $n = 17$; at 6 mos, SPI+: $n = 18$; SPI- $n = 19$, MPI: $n = 18$

Table 8: Antigen expression

HSCORE	SPI+ n = 14	SPI- n = 14	MPI n = 13	
Bax				
Baseline	1.38 ± 0.08	1.45 ± 0.07	1.35 ± 0.06	0.6131
6 Mos	1.41 ± 0.06 *ab*	*1.27 ± 0.05 *a*	1.44 ± 0.06 *b*	0.0818
PCNA				
Baseline	1.61 ± 0.1	1.93 ± 0.1	1.86 ± 0.1	0.1494
6 Mos	1.69 ± 0.1	*1.57 ± 0.1	1.81 ± 0.1	0.4107
Bcl-2				
Baseline	1.11± 0.03	*1.17 ± 0.07	1.09 ± 0.03	0.4629

6 Mos	1.15 ± 0.04	*1.15 ± 0.04	1.19 ± 0.04	0.7195
EGFr				
Baseline	1.34 ± 0.08	1.42 ± 0.10	1.39 ± 0.11	0.8264
6 Mos	1.36 ± 0.06	1.37 ± 0.06	1.33 ± 0.06	0.8342
Bax: Bcl-2 ratio				
Baseline	1.25 ± 0.07	1.30 ± 0.10	1.23 ± 0.06	0.8559
6 Mos	1.20 ± 0.05	1.14 ± 0.05	1.22 ± 0.05	0.4806
Bax: PCNA ratio				
Baseline	0.875 ± 0.05	0.758 ± 0.05	0.760 ± 0.05	0.1826
6 Mos	0.894 ± 0.05	0.823 ± 0.05	0.839 ± 0.05	0.6111

Baseline data are unadjusted means ± standard errors. All other data are least-squares means adjusted for baseline measurement ± standard errors. Pre-planned treatment pairwise comparisons are between groups within each row: means that do not share letters are significantly different ($p < 0.05$).

The number of patients evaluated for bax expression was 16 for SPI-, and 14 for MPI;

PCNA expression was 13 for SPI-, and 12 for MPI; Bcl-2 expression was 16 for MPI; EGFr expression was 15 for SPI+; Bax: bcl-2 ratio was 13 for SPI+; Bax: PCNA ratio was 13 for SPI+, 13 for SPI-, and 12 for MPI.

Cancer Incidence: The incidence of prostate cancer (6% in the SPI (+) group, 6% in the SPI (-) group and 38% in the MPI group) was more than 6 times higher in the MPI versus both soy groups (*P* = 0.013).

Effect of equol excretor status on serum hormones and urinary estrogen metabolites: Individuals whose urinary equol concentration exceeded 1000 nmol/day were classified as equol excretors. At 3-months, 4 individuals were classified as equol excretors and 15 as non-excretors in the SPI (+) group. However, at 6-months, only one of these four individuals remained as an equol excretor. Hence comparisons between equol-excretors and non-excretors were only made at the 3-months time-point.

No baseline differences in anthropometrics, dietary intake and cancer status were observed between equol excretors and non-excretors. However, baseline urinary 2:16 OH-E1 concentrations tended to be higher in excretors. After 3-months of SPI (+) intake, serum hormone concentrations and urinary estrogen metabolite levels did not differ between equol excretors and non-excretors.

Data for the above endpoints and manuscripts have been published.

KEY RESEARCH ACCOMPLISHMENTS

Effects of SPI (+):

- No effects on circulating hormone concentrations
- Decreased serum SHBG levels
- Decreased prostatic androgen receptor expression, no effect on estrogen-receptor beta expression
- Increased 24-hour urinary estradiol and concentrations
- Higher 2:16 OH estrone ratio as compared to the MPI group
- No effect on prostate cancer tissue biomarker

Although SPI (+) had no effects on circulating hormone concentrations and decreased SHBG levels (which would theoretically increase androgen availability), prostatic AR expression was lowered. Additionally, increases in urinary estradiol and estrone concentrations and an elevation in the 2:16-OH estrone ratio have been associated with reduced prostate cancer risk. Overall, the effects observed with the SPI (+) interventions are consistent with a protective effect of SPI (+) against prostate cancer.

Effects of SPI (-)

- Increased circulating androgen (androstenedione, DHEAS) and estrogen (estradiol, estrone) concentrations
- Tended to decrease androgen receptor expression, no effects on estrogen-receptor beta expression.
- Decreased serum SHBG levels
- Increased 24-hour urinary estradiol and estrone concentrations
- Reduced prostatic Bax (a protein that is pro-apoptotic) and PCNA (a protein which is a marker of cell proliferation) expression.

Effects of SPI (-) on study endpoints were mixed, with some considered detrimental (reduced Bax expression, decreased serum SHBG concentrations, increases in circulating androgen concentrations) and others beneficial (decreases in PCNA expression, increases in urinary estrone and estradiol levels). It is important to note that although circulating levels of androstenedione and DHEAS increased, serum testosterone levels remained unchanged.Also, prostatic AR expression tended to decrease. Overall, the effects observed with the SPI (-) intervention are consistent with a neutral effect of SPI (-) on prostate cancer prevention.

Effects of MPI:

- No effects on circulating hormone concentration
- Decreased serum SHBG level
- Decreased urinary 2-OH estradiol concentrations
- No effects on prostatic androgen receptor and estrogen-receptor beta expression

- No effect on prostate cancer tissue biomarker

Consistent with its use as a control, most study endpoints were unaltered with the MPI intervention. Decreases in serum SHBG were also observed in the soy groups and were likely due to the increased protein intake observed in all 3 groups.

REPORTABLE OUTCOMES

Jill M. Hamilton-Reeves obtained her PhD in December of 2006.

Jill Hamilton-Reeves won the **Clinical Young Investigator Award** (2007) sponsored by the American Society for Nutrition for outstanding writing and oral presentation.

Jill Hamilton-Reeves **won the Coca-Cola Company Research Award** (2007) sponsored by the Diet & Cancer Research Interest Section of the American Society for Nutrition for excellent research quality and technical presentation .

Abstracts presented:

Federation of American Societies for Experimental Biology (FASEB)
April 2007; Washington DC
Oral Presentation: “Soy and Estrogen Metabolites in Men”

Innovative Minds in Prostate Cancer Today (IMPaCT)
September 2007; Atlanta, GA
Poster Presentation: “Soy and Estrogen Metabolites in Men”

Publications:

JM Hamilton-Reeves, SA Rebello, W Thomas, JW Slaton, and MS Kurzer. Isoflavone-rich soy protein isolate suppresses androgen receptor expression without altering estrogen receptor beta expression or serum hormonal profiles in men at high risk of prostate cancer. *Journal of Nutrition*. 2007 137:1769-1775.

JM Hamilton-Reeves, SA Rebello, W Thomas, JW Slaton, and MS Kurzer. Soy protein isolate increases urinary estrogens and the ratio of 2:16α-hydroxyestrone in men at high risk of prostate cancer. *Journal of Nutrition.* 2007 137:2258-2263.

JM Hamilton-Reeves, SA Rebello, W Thomas, JW Slaton, and MS Kurzer. Effects of soy protein isolate consumption on prostate cancer biomarkers in men at high risk of prostate cancer. *Nutrition and Cancer. 2008 60:(1) 7 - 13.*

Lay publication:
Hamilton-Reeves J and Kurzer MS. (2003) Effects of soy isoflavone consumption on reproductive hormones in males. *Soy Connection* 11(4): 3-5.

CONCLUSIONS

The objective of this project was to evaluate the effects of isoflavone-rich soy protein isolate on prostate cancer biomarkers in men at high risk of cancer and to determine whether or not isoflavones are the responsible bioactive components of soy. Isoflavone-rich soy protein isolate suppressed androgen receptor density, increased urinary estrogen excretion, and increased the 2:16 OH-E1 ratio in the urine. Similarly, isoflavone-poor soy protein isolate tended to lower androgen receptor density, and it significantly increased urinary estrogen excretion. Moreover, the isoflavone-poor soy protein isolate increased serum estradiol and androstenedione concentrations, and showed mixed effects on prostate tissue markers. Interestingly, we observed a trend toward a lower rate of prostate cancer development in the men in the soy groups compared to the men in the milk group. Taken together, these findings suggest that soy protein isolate mediates prostate cancer preventive effects in men at high risk of developing prostate cancer. However, it is unclear whether other soy constituents were responsible for the effects, or if the low level of isoflavones in the isoflavone-poor soy protein isolate were sufficient to exert the observed effects. This suggests that a larger phase III clinical trial of soy protein in men at high risk of prostate cancer is warranted, with cancer as an outcome.

REFERENCES

None

APPENDICES

Published peer-reviewed works from reportable outcomes in order of publication are below.

The Journal of Nutrition
Nutrition and Disease

Isoflavone-Rich Soy Protein Isolate Suppresses Androgen Receptor Expression without Altering Estrogen Receptor-β Expression or Serum Hormonal Profiles in Men at High Risk of Prostate Cancer[1–3]

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Abstract

The purpose of this study was to determine the effects of soy protein isolate consumption on circulating hormone profiles and hormone receptor expression patterns in men at high risk for developing advanced prostate cancer. Fifty-eight men were randomly assigned to consume 1 of 3 protein isolates containing 40 g/d protein: *1*) soy protein isolate (SPI+) (107 mg/d isoflavones); *2*) alcohol-washed soy protein isolate (SPI−) (<6 mg/d isoflavones); or *3*) milk protein isolate (0 mg/d isoflavones). For 6 mo, the men consumed the protein isolates in divided doses twice daily as a partial meal replacement. Serum samples collected at 0, 3, and 6 mo were analyzed for circulating estradiol, estrone, sex hormone-binding globulin, androstenedione, androstanediol glucuronide, dehydroepiandrosterone sulfate, dihydrotestosterone, testosterone, and free testosterone concentrations by RIA. Prostate biopsy samples obtained pre- and postintervention were analyzed for androgen receptor (AR) and estrogen receptor-β expression by immunohistochemistry. At 6 mo, consumption of SPI+ significantly suppressed AR expression but did not alter estrogen receptor-β expression or circulating hormones. Consumption of SPI− significantly increased estradiol and androstenedione concentrations, and tended to suppress AR expression ($P = 0.09$). Although the effects of SPI− consumption on estradiol and androstenedione are difficult to interpret and the clinical relevance is uncertain, these data show that AR expression in the prostate is suppressed by soy protein isolate consumption, which may be beneficial in preventing prostate cancer. J. Nutr. 137: 1769–1775, 2007.

Introduction

Steroid hormones modulate growth of the prostate gland, and elevated levels of androgens have been associated with prostate cancer risk (1,2). Consumption of soy foods is thought to contribute to prostate cancer prevention as a result of the hormonal properties of soy isoflavones, either through altered endogenous circulating hormones or hormone-receptor signaling. Cell culture studies have suggested that the isoflavonoids, genistein and equol, exert the most noteworthy hormonal effects. Genistein inhibits the activity of 5α-reductase and 17β-hydroxysteroid dehydrogenase, enzymes required for androgen synthesis (3,4). The isoflavonoid equol, a bacterially derived metabolite of the isoflavone daidzein, sequesters dihydrotestosterone (DHT)[8] from the androgen receptor (AR) in rat prostate tissue (5). Both isoflavonoids accumulate in the prostate gland (6–9) and may mimic or modulate endogenous hormones relevant to prostate carcinogenesis.

Despite evidence from in vitro studies, human intervention studies report inconsistent effects of soy or isoflavone consumption on circulating hormone profiles in men. Although reports show statistically significant suppression of total testosterone (10,11), sex hormone binding globulin (SHBG) (12), DHT (13), dehydroepiandrosterone (14), estrone (15), and free androgen index (13), and increased concentrations of SHBG (16) and DHT (17), the majority of the 22 intervention studies to date have not found significant changes in circulating sex steroid hormones (10–31). Generally, the studies that report significant changes were

[1] The Soy and Prostate Cancer Prevention (SoyCaP) trial was supported by grant DAMD 17-02-1-0101 (M.S.K.) and W81XWH-06-1-0075 (J.H.R.) from the United States Army Department of Defense Prostate Cancer Research Program. The protein isolates were donated by The Solae Company, St. Louis, MO. Neither sponsor was involved in writing this report.

[2] Author disclosures: J. M. Hamilton-Reeves, S. A. Rebello, W. Thomas, J. W. Slaton, and M. S. Kurzer, no conflicts of interest.

[3] Supplemental Tables 1 and 2 are available with the online posting of this paper at jn.nutrition.org.

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[8] Abbreviations used: 3α-AG, androstanediol glucuronide; AR, androgen receptor; DHEAS, dehydroepiandrosterone sulfate; DHT, dihydrotestosterone; ERβ, estrogen receptor-β; MPI, milk protein isolate; SHBG, sex hormone-binding globulin; SPI−, alcohol-extracted soy protein isolate; SPI+, isoflavone rich soy protein isolate.

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carried out in older men for a relatively long duration. None of the published studies reported equol-excretor status effects on circulating hormone response to soy isoflavone interventions in men.

Circulating hormone profiles may fail to accurately reflect prostate tissue exposure, and evaluating hormone receptor expression patterns in the prostate may provide additional evidence concerning the role of soy as a cancer preventive dietary agent. The AR mediates the action of androgens, and AR expression is a potential marker for prostate cancer prognosis (32). Dietary genistein has been shown to downregulate AR mRNA expression in rodents (33,34), and genistein has been shown to suppress AR activity through an estrogen receptor-β (ERβ)-dependent mechanism in LNCaP cells (35). Despite these data, to our knowledge, there are no studies published to date that evaluate the effects of soy protein isolate consumption on AR and ERβ expression in men, although one study reported that an isoflavone extract derived from red clover failed to alter AR expression compared with historically matched controls (26).

The objective of this project was to evaluate the effects of isoflavone-rich soy protein isolate consumption on circulating concentrations of reproductive hormones and prostate tissue markers of estrogen and androgen receptor expression in men at high risk of prostate cancer. The effects of an isoflavone-rich soy protein isolate were compared with those of an isoflavone-poor soy protein isolate to determine whether the isoflavones are the responsible bioactive constituents. The underlying hypothesis was that isoflavone-rich soy protein isolate consumption would reduce circulating hormones, downregulate AR expression, and upregulate ERβ expression.

Material and Methods

Subjects. Fifty-eight men, aged 50–85 y, were recruited at the Minneapolis Veteran's Administration Medical Center Urology Clinic from a group of patients that had already undergone a transrectal ultrasound and biopsy. Patients in this study were either at high risk for developing prostate cancer ($n = 53$), or had low-grade prostate cancer that was being followed by active surveillance ($n = 5$). Subjects were considered high risk if they had high-grade prostatic intraepithelial neoplasia (PIN) ($n = 50$) and/or atypical small acinar proliferation (ASAP) ($n = 14$). The subjects with prostate cancer had Gleason scores of <6 and were not receiving any other prostate cancer therapy. Subjects were recruited by urologic physicians, and the research nurse reviewed the patients' medical records to determine that eligibility criteria were met. Exclusionary criteria included BMI >40 kg/m^2, prostate cancer that required medical treatment, prostatitis, alcohol consumption >14 drinks/wk, soy or milk allergy, regular antibiotic use, or renal insufficiency.

Eighty-seven subjects were screened for the study; 21 chose not to participate after attending the orientation session, and 66 subjects began the study. Eight subjects withdrew from the study before their 3-mo appointment [disliked the study treatment powder ($n = 3$), inconvenienced by study demands ($n = 2$), gastrointestinal discomfort ($n = 1$), chose conventional prostate cancer treatment ($n = 1$), weight gain ($n = 1$)]. Three subjects completed 3 mo of the study with good compliance but chose not to finish due to inconvenience of the study demands, and 55 subjects completed the full 6-mo study.

Data from 58 subjects were included in the serum hormone analysis, and 42 subjects were included in the hormone receptor expression analysis. Fewer participants were eligible for the hormone expression analysis because 3 subjects did not undergo the final prostate biopsy [liver cancer diagnosis ($n = 1$), heart condition ($n = 1$), not clinically indicated ($n = 1$)], and 13 subjects had insufficient biopsy tissue at either baseline or postintervention for the analyses. All 58 subjects who completed the study were Caucasian.

Study design. The University of Minnesota Institutional Review Board Human Subjects Committee, the Minneapolis Veterans Affairs Institutional Review Board, and the U.S. Army Medical Research and Materiel Command's Human Subjects Research Review Board approved the study protocol, and all subjects provided informed consent, attended an orientation session, and were provided with a study handbook. During the study orientation, subjects were interviewed and prompted about incidental exposure to dietary isoflavones (e.g., snack bars, shakes, soy nuts, canned tuna, legumes, breads) to determine whether they were soy consumers. Only one participant reported regular soy consumption, but he did not consume soy-containing products for 1 mo prior to beginning the study. The 6-mo intervention study used a randomized, single-blinded, placebo-controlled, parallel design. Free-living subjects supplemented their diets with 1 of 3 randomly assigned protein isolates: *1*) soy protein isolate high in isoflavones (SPI+); *2*) soy protein isolate that had most of the isoflavones removed by alcohol extraction (SPI−); or *3*) milk protein isolate (MPI) (The Solae Company). The protein isolates were consumed in divided doses twice daily and contributed 40 g/d protein and 200–400 kcal/d (1 kcal = 4.184 kJ). The isoflavone content of the protein isolates expressed as aglycone equivalents was 107 ± 5.0 mg/d for the SPI+; <6 ± 0.7 mg/d for the SPI−; and 0 mg/d for the MPI (mean ± SD). The mean distribution of isoflavones was 53% genistein, 35% daidzein, and 11% glycitein in SPI+, and 57% genistein, 20% daidzein, and 23% glycitein in SPI− as analyzed by Dr. Pat Murphy (Department of Food Science and Human Nutrition, Iowa State University). The packets of protein isolate were numbered and patients were unaware of the treatment protein isolate they had been assigned until all subjects completed the intervention. Only the study coordinators who administered the protein isolates knew the group to which each participant belonged. Compliance was assessed by counting the number of times the patient consumed the protein isolate, as self-reported in recording calendars given to them, and mean compliance was 94%. Dietary and herbal supplements were allowed, and participants were asked to avoid changing dosages or adding new supplements to their regimen during the study. Subjects consumed their habitual diets, and received detailed instructions to exclude soy products to minimize isoflavone consumption from other sources.

Serum collection and analysis. Fasting blood was collected in the morning at 0, 3, and 6 mo. Serum was separated and aliquots were frozen at –70°C until analysis. All serum samples were analyzed for testosterone, free testosterone, DHT, androstanediol glucuronide (3α-AG), androstenedione, dehydroepiandrosterone sulfate (DHEAS), SHBG, estradiol, and estrone. Steroid hormones were analyzed in duplicate by RIA, and SHBG was analyzed by immunoradiometric assay (Diagnostics Systems Laboratories). Hormone analyses were performed in 3 batches and all assays required ^{125}I-labeled analyte. Intraassay variabilities were 3.7% for testosterone, 4.4% for free testosterone, 6.1% for DHT, 4.5% for 3α-AG, 4.4% for androstenedione, 2.3% for DHEAS, 4.4% for SHBG, 3.9% for estradiol, and 4.3% for estrone. An internal control was utilized to determine variability among batches, and interassay variabilities were between 9 and 30% for all analytes. All 3 serum samples for each participant were analyzed in the same batch.

Urine collection and analysis. To assess equol-producer status, 24-h urine was collected in plastic containers containing 1 g/L of ascorbic acid and separated into aliquots after the addition of sodium azide to a final concentration of 0.1%. Aliquots were frozen at –20°C until analysis. Equol was determined by HPLC and MS as previously described (36). The intraassay CV for equol was 8.2%, and the interassay CV was 12.5%. Subjects were classified as equol excretors if 24-h urine equol levels exceeded 1000 nmol/d.

Dietary intake and analysis. Food records were completed for 3 d before each clinic visit. A registered dietitian taught study participants how to keep accurate food records. Patients were encouraged to use household scales and volumetric tools and to submit food labels from unusual foods. Study coordinators reviewed each food record for completeness and clarified ambiguities with the participant at each clinic visit. Food records were analyzed with Nutritionist V, version 2.3 (37), and, for each 3-d food record, mean intakes of energy, macronutrients, saturated fat, cholesterol, fiber, vitamin D, vitamin E, calcium, selenium, and zinc were calculated.

Tissue collection and analysis. Biopsies were performed before the initial screening and at the 6-mo clinical visit. Biopsy cores were formalin-preserved for 24 h and paraffin embedded. The histological diagnoses were determined during a routine pathological evaluation. Immunohistochemistry was performed to assess AR and ERβ expression on primarily normal, hyperplastic, or preneoplastic glands collected from eligible study participants. Antigen retrieval was achieved by pressure cooking deparaffinized and rehydrated tissue sections at 103 kPa in citrate buffer. Sections were treated in quenching solution (3% H_2O_2 in 100% methanol), and then incubated with a protein-blocking solution (10% milk, 5% serum, and 1% BSA). Samples were incubated overnight at 4°C with rabbit polyclonal anti-ERβ antibody (ab3577; Abcam; 1:1000) for the ERβ assay, or for 30 min at room temperature with the mouse monoclonal anti-AR antibody (AM256–2M; BioGenex; RTU) for the AR assays. Next, the avidin-biotin peroxidase method was carried out (Vectastain Elite ABC kit, Vector Laboratories). Color reaction was developed using diaminobenzidine as the chromagen. Appropriate positive and negative controls were included in all staining runs. Disrupted glands and glands on the edge of tissue sections were excluded from analysis to avoid false positives. A technician without prior knowledge of histological grading scored both the intensity of immunostaining and the percentage of immunopositive areas at 40× magnification using the HSCORE system as previously described (38). The range of the HSCORE is a minimum of 1 and a maximum of 4 (1 indicated absent staining; 4 indicated intense staining). A mean of 6 intact glands (range: 2–15) per slide for ERβ and a mean of 8 intact glands (range: 3–19) per slide for AR were averaged to derive the HSCORE (**Fig. 1**).

Excluded from analysis. The following data were excluded from statistical analysis: 6-mo dietary intake from one participant reporting unusually low consumption (mean <500 kcal) (1 kcal = 4.184 kJ) during the 3-d food diary as a result of illness; 3 mo DHEAS that was above normal range (16 μmol/L) and inconsistent with the participant's baseline and 6-mo measurements; all DHEAS measurements from one participant with abnormally high 3-mo and 6-mo DHEAS concentrations (9 and 10 μmol/L, respectively) compared with baseline; and all SHBG measurements from one subject with undetectable SHBG in the serum (<3 nmol/L). One subject did not consume the treatment powder for 3 d prior to his 6-mo appointment as a result of illness, so he was excluded from the 6-mo equol excretion analysis.

Statistical analysis. The data appeared normally distributed and had similar variance among groups. Demographic comparisons between groups were performed with 1-way ANOVA for continuous endpoints, and chi-square for categories of prostate cancer markers. ANCOVA was used to compare groups adjusted for the baseline value of the final endpoint. For androstenedione, the model included a treatment by baseline interaction. Preplanned pairwise comparisons of all groups are reported for each endpoint as dictated by the study hypotheses: each group's adjusted mean (least squares mean) was compared with the other 2 groups' adjusted means. Paired *t* tests were used to test for significant within-group changes over time. In addition, these covariates were screened as adjusters: baseline body weight, equol excretor status, and energy and nutrient intake. $P < 0.05$ was considered significant. All analyses were performed using SAS, version 9.1 (39).

Results

Baseline. Baseline anthropometrics, cancer status, and dietary intake did not differ among the groups (**Table 1**), except that the MPI group had a higher body weight and the SPI− group consumed significantly more protein, calcium, and zinc at baseline (**Table 2**). Baseline prostate steroid receptor expression patterns (**Table 3**) and serum hormone and SHBG concentrations (**Table 4**) did not differ among the groups.

Anthropometrics and dietary intake. Body weight did not change from baseline to 3 or 6 mo in any group (Table 2), and the significant differences in body weight among the groups at baseline were maintained. Protein, calcium, and vitamin D intakes increased in all groups during the study as a result of their concentrations in the protein isolates, and the differences in protein, calcium, and zinc intake at baseline were not present at 3 and 6 mo. At 3 mo, total and saturated fat consumption were reduced in the SPI− group relative to baseline. During the study, energy, carbohydrate, cholesterol, fiber, vitamin E, selenium, and zinc intakes did not change for any group. Dietary and herbal supplement usage did not differ among groups (data not shown). Body weight and protein intake differences among groups were unrelated to altered hormone concentrations or steroid receptor expression patterns.

Steroid receptors. Baseline-adjusted AR expression was lower in prostate biopsies after 6 mo in the SPI+ group compared with the MPI group ($P = 0.04$) and tended to be lower in the SPI− group compared with the MPI group ($P = 0.09$; Table 3). AR expression significantly increased from baseline in the MPI group, but not in the other 2 groups. There were no changes from baseline in ERβ expression among the groups (Table 3).

Serum estrogens. The serum estradiol concentration was significantly increased in the SPI− group at 3 and 6 mo relative to baseline, and by 6 mo, baseline-adjusted estradiol concentrations were significantly higher in the SPI− group compared with the other 2 groups (Table 4). Serum estrone was also significantly increased in the SPI− group at 3 and 6 mo, and was significantly higher than in the MPI group at 3 mo but not at 6 mo.

Serum androgens and SHBG. The serum androstenedione concentration was significantly higher in the SPI+ group than in the MPI group at 3 mo. At 6 mo it was significantly greater than at baseline in the SPI− group, resulting in a significantly higher concentration than in the SPI+ group (Table 4). At both 3 and 6 mo, serum DHEAS was higher in the SPI group than in the other 2 groups, and at 3 mo, 3α-AG was higher in the SPI− group than the other 2 groups. At 3 mo, the DHT concentration decreased from baseline in the SPI group. Serum SHBG concentrations were decreased significantly from baseline at 3 and 6 mo in all groups, with no difference among the groups.

Equol-excretor status and hormone profiles. Equol excretor status was assessed only in the SPI+ group, because only they consumed sufficient daidzein to excrete equol. At 3 mo, there were 4 excretors and 15 nonexcretors, but of this group, only 1 excretor remained at 6 mo [dropped out after 3 mo ($n = 1$),

TABLE 1 Baseline characteristics of subjects[1]

	SPI+	SPI−	MPI
n	20	20	18
Age, *y*	68 ± 8	68 ± 5	68 ± 7
Body wt, *kg*	91 ± 16[ab]	88 ± 12[a]	98 ± 15[b]
Height, *cm*	175 ± 7	173 ± 8	176 ± 8
BMI, *kg/m²*	30 ± 5	29 ± 4	32 ± 6
Prostate cancer markers,[2] *n (%)*			
PIN	15 (75)	12 (60)	13 (72)
ASAP	3 (15)	7 (35)	3 (17)
CaP	2 (10)	1 (5)	2 (11)

[1] Values are means ± SD or *n* (%). Means in a row with superscripts without a common letter differ, $P < 0.05$.
[2] Subjects were categorized by most advanced prostate cancer marker.

TABLE 2 Anthropometrics and dietary intake of men at high risk of prostate cancer that consumed various protein isolates for 6 mo[1]

	SPI+	SPI−	MPI
n[2]	20	20	18
Weight, *kg*			
Baseline	91 ± 16[ab]	88 ± 12[a]	98 ± 15[b]
3	91 ± 16[ab]	87 ± 12[a]	98 ± 15[b]
6	90 ± 16[ab]	87 ± 13[a]	99 ± 15[b]
Height, *cm*			
Baseline	175 ± 16	173 ± 8	176 ± 8
BMI, *kg/m*^{2}			
Baseline	30 ± 5	29 ± 4	32 ± 6
3	30 ± 5	29 ± 4	32 ± 6
6	30 ± 5	29 ± 4	32 ± 6
Energy intake,[3] *kcal/d*			
Baseline	2140 ± 620	2260 ± 660	2070 ± 520
3	2220 ± 720	2030 ± 390	2180 ± 510
6	2240 ± 410	2120 ± 670	2330 ± 410
Protein, *g/d*			
Baseline	83 ± 21[a]	100 ± 24[b]	81 ± 25[a]
3 Mo	*118 ± 24	*117 ± 16	*121 ± 30
6 Mo	*118 ± 21	*124 ± 29	*120 ± 18
Carbohydrate, *g/d*			
Baseline	256 ± 106	262 ± 118	236 ± 59
3 Mo	246 ± 97	230 ± 82	232 ± 75
6 Mo	251 ± 61	232 ± 89	256 ± 68
Total fat, *g/d*			
Baseline	86 ± 33	93 ± 32	88 ± 24
3 Mo	80 ± 39	*74 ± 18	73 ± 30
6 Mo	83 ± 34	80 ± 34	89 ± 26
Saturated fat, *g/d*			
Baseline	27 ± 11	34 ± 14	28 ± 11
3 Mo	27 ± 13	*26 ± 7	24 ± 12
6 Mo	26 ± 10	29 ± 14	30 ± 10
Cholesterol, *mg/d*			
Baseline	324 ± 202	382 ± 153	301 ± 163
3 Mo	307 ± 131	296 ± 115	312 ± 233
6 Mo	328 ± 147	348 ± 175	329 ± 234
Fiber, *g/d*			
Baseline	17 ± 9	18 ± 7	16 ± 5
3 Mo	16 ± 8	17 ± 8	15 ± 7
6 Mo	15 ± 9	16 ± 9	15 ± 5
Vitamin D, μ*g/d*			
Baseline	4 ± 3	4 ± 5	4 ± 3
3 Mo	*9 ± 4	*8 ± 3	*8 ± 2
6 Mo	*8 ± 2	*8 ± 3	*9 ± 2
Vitamin E, *mg/d*			
Baseline	8 ± 7	8 ± 5	6 ± 4
3 Mo	6 ± 4	7 ± 10	6 ± 3
6 Mo	7 ± 7	6 ± 3	6 ± 3
Calcium, *mg/d*			
Baseline	890 ± 400[ab]	1230 ± 970[b]	760 ± 360[a]
3 Mo	*2260 ± 440	*2120 ± 350	*2200 ± 380
6 Mo	*2180 ± 290	*2340 ± 840	*2190 ± 340
Selenium, *mg/d*			
Baseline	0.08 ± 0.05	0.09 ± 0.03	0.08 ± 0.05
3 Mo	0.08 ± 0.03	*0.06 ± 0.03	0.10 ± 0.11
6 Mo	0.07 ± 0.03	*0.07 ± 0.02	0.44 ± 1.6
Zinc, *mg/d*			
Baseline	10 ± 6[a]	14 ± 5[b]	10 ± 5[a]
3 Mo	11 ± 4	10 ± 8	10 ± 3
6 Mo	9 ± 3	10 ± 5	9 ± 3

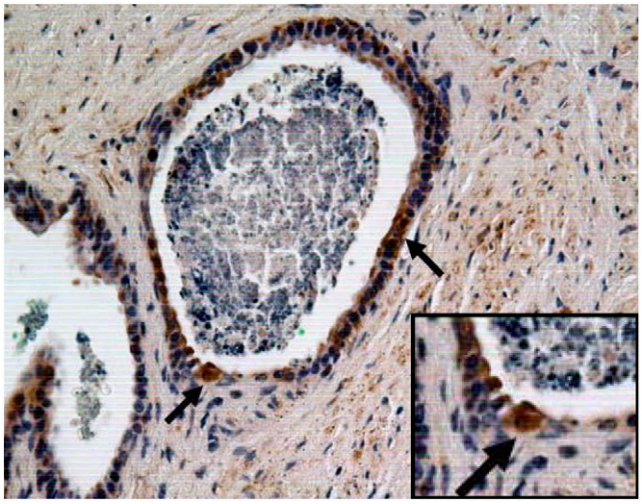

FIGURE 1 Representative immunohistochemical staining of AR in human prostate core biopsies for HSCORE. Arrow indicates stained acinar cell in MPI control group (enlarged view inset in lower right).

apparently changed status ($n = 1$), and excluded data ($n = 1$)]. Baseline characteristics (**Supplemental Table 1**) and serum hormone concentrations at 3 mo (**Supplemental Table 2**) did not differ between excretors and nonexcretors.

Discussion

The present study evaluated men at high risk of prostate cancer to determine the effects of soy protein consumption on serum hormones and prostate tissue steroid receptor expression levels. The major finding was lower AR expression levels and no differences in ERβ expression or circulating hormones in men consuming SPI+ compared with those consuming MPI.

AR increased significantly from baseline in the MPI group, but did not change from baseline in the soy groups. Because AR expression is expected to increase in this population (40), we infer that SPI+ apparently prevented or suppressed a rise in AR expression. Lower tissue AR expression in the SPI+ group is consistent with research in which dietary phytoestrogens downregulated AR mRNA expression in adult male rats (33,34,41). Our data differ, however, from those of Jarred et al. (26), who reported no differences in AR expression patterns between radical prostatectomy patients treated with isoflavones and historically matched controls. The inconsistent results between the 2 studies can be explained by several methodological differences. In the study by Jarred et al. (26), the subjects, who consumed 160 mg/d of isoflavones in extracts derived from red clover, were men with advanced prostatic neoplasms treated for short and varied time periods (7–54 d). The tissue sections studied from the radical prostatectomies taken from treated subjects represented cancerous glandular acinae and were compared with sections of cancers from historically matched controls. Our subjects consumed 107 mg/d of isoflavones in isoflavone-rich SPI, were earlier in the carcinogenesis continuum, were treated

TABLE 2 *Continued*

[1] All values are means ± SD. Means in a row with superscripts without a common letter differ, $P < 0.05$. *Different from baseline, $P < 0.05$.
[2] Sample sizes are for all time points except the following: 3 mo, MPI ($n = 17$), and 6 mo, SPI+ ($n = 18$), and SPI− ($n = 18$).
[3] 1 kcal = 4.184 kJ.

TABLE 3 Steroid receptor expression of men at high risk of prostate cancer that consumed various protein isolates for 6 mo[1]

	SPI+	SPI−	MPI
Androgen receptor		*HSCORE*	
n	14	16	14
Baseline	1.37 ± 0.06	1.28 ± 0.06	1.23 ± 0.06
6 Mo	1.26 ± 0.05[a]	1.30 ± 0.05[ab]	*1.42 ± 0.05[b]
Estrogen receptor-β			
n	14	14	15
Baseline	1.22 ± 0.06	1.32 ± 0.06	1.23 ± 0.06
6 Mo	1.16 ± 0.06	1.18 ± 0.06	1.26 ± 0.05

[1] Baseline data are unadjusted means ± SEM. All other data are least-squares means adjusted for baseline measurement ± SEM. Means in a row with superscripts without a common letter differ, $P < 0.05$. *Different from baseline, $P < 0.05$.

TABLE 4 Serum hormones and SHBG in men at high risk of prostate cancer that consumed various protein isolates for 6 mo[1]

	SPI+	SPI−	MPI
n[2]	20	20	18
Estradiol, *pmol/L*			
Baseline	67 ± 4	66 ± 4	69 ± 3
3 Mo	75 ± 5	*76 ± 5	*62 ± 6
6 Mo	69 ± 3[a]	*79 ± 3[b]	66 ± 3[a]
Estrone, *pmol/L*			
Baseline	157 ± 15	141 ± 10	158 ± 8
3 Mo	150 ± 8[ab]	*170 ± 8[b]	146 ± 8[a]
6 Mo	152 ± 10	*171 ± 10	150 ± 10
Androstenedione, *nmol/L*			
Baseline	2.9 ± 0.3	2.9 ± 0.3	2.5 ± 0.2
3 Mo	3.0 ± 0.2[a]	3.0 ± 0.2[ab]	2.8 ± 0.2[b]
6 Mo	2.6 ± 0.2[a]	*3.4 ± 0.2[b]	2.9 ± 0.2[ab]
Androstanediol glucuronide, *nmol/L*			
Baseline	19 ± 3	18 ± 5	16 ± 2
3 Mo	17 ± 2[a]	24 ± 2[b]	17 ± 2[a]
6 Mo	16 ± 2	20 ± 2	18 ± 2
DHEAS,[3] *nmol/L*			
Baseline	2202 ± 390	2052 ± 300	1977 ± 370
3 Mo	2040 ± 103[a]	2715 ± 103[b]	2126 ± 103[a]
6 Mo	1937 ± 154[a]	2372 ± 146[b]	1946 ± 150[a]
DHT, *pmol/L*			
Baseline	1547 ± 190	1354 ± 170	1072 ± 110
3 Mo	1242 ± 81	*1076 ± 79	1119 ± 100
6 Mo	1215 ± 94	1174 ± 89	1229 ± 105
Testosterone, *nmol/L*			
Baseline	12 ± 1	13 ± 1	12 ± 1
3 Mo	13 ± 0.5	13 ± 0.6	11 ± 0.6
6 Mo	13 ± 0.6	13 ± 0.5	12 ± 0.6
Free testosterone, *pmol/L*			
Baseline	33 ± 3	34 ± 2	29 ± 2
3 Mo	33 ± 1	33 ± 1	32 ± 1
6 Mo	32 ± 1	32 ± 1	31 ± 1
SHBG,[4] *nmol/L*			
Baseline	63 ± 7	64 ± 8	69 ± 9
3 Mo	*56 ± 3	*56 ± 2	*56 ± 3
6 Mo	*54 ± 3	*61 ± 3	*58 ± 3

[1] Baseline data are unadjusted means ± SEM. All other data are least-squares means adjusted for baseline measurement ± SEM, except androstenedione, which is additionally adjusted for interaction between treatment and baseline. Means in a row with superscripts without a common letter differ, $P < 0.05$. *Different from baseline, $P < 0.05$.
[2] Sample sizes are for all time points except: 3 mo MPI ($n = 17$), and 6 mo SPI+ ($n = 18$), and SPI− ($n = 19$).
[3] Sample sizes differed from other hormones due to excluded data. At 3 mo, SPI+ ($n = 19$) and SPI− ($n = 19$). At 6 mo, SPI+ ($n = 17$) and SPI− ($n = 19$).
[4] Sample sizes differed from other hormones due to excluded data. At 3 mo, SPI+ ($n = 19$), and at 6 mo, SPI+ ($n = 18$).

for 6 mo each, and all biological samples were evaluated within the same subject before and after the intervention. Furthermore, the gland acinae studied presented either benign, hyperplastic, or preneoplastic tissue.

Consumption of SPI+ did not affect ERβ expression or circulating hormones. The ERβ expression results are inconsistent with studies in animals in which prolonged isoflavone exposure decreased ERβ expression (33,42), and may be explained by the variability in commercially available ERβ antibodies (43). Our hormone results, however, are consistent with most published reports from the clinical setting. The testosterone results are consistent with numerous soy or isoflavone intervention studies in which no change in total testosterone was observed (12–31), but differ from 2 studies of short duration (10,11). Our finding of no effect on directly measured free testosterone is similar to published soy or isoflavone intervention studies to date (11,14,15,20,22,24), and our finding of no effect on circulating DHT is consistent with most reports (10,14,16,19–21,23,30), although it differs from results of 2 studies (13,17), one of which used red clover extract (17). The lack of effect on circulating estradiol or estrone is consistent with the literature (10,11,15,16,19,22,29,30), although there is one report of decreased estrone in men consuming soymilk for 8 wk (15).

Serum SHBG decreased significantly from baseline in all study groups. The finding that consumption of SPI+ decreased SHBG is similar to a report by Mackey et al. (12); however, they did not find a significant decrease in SHBG with an isoflavone-poor protein isolate as we did. In contrast to our findings, Habito et al. (16) reported increased SHBG in men consuming 35 g of tofu daily for 2 wk, and others have reported no significant changes of SHBG with isoflavone-rich foods or extracts (13,15,17,20–23,30). Decreased SHBG is a potentially harmful effect because SHBG-bound hormones are less biologically available to stimulate hormone-sensitive cancers. Because high protein intake has been associated with decreased SHBG (44), it is likely that the decrease in SHBG from baseline in all groups in our study resulted from the subjects' significantly increased protein intake during the study (45).

The hormonal effects in the SPI− group were unexpected. Although AR expression was not significantly lower in the SPI− group, AR expression appeared to be intermediate between that of SPI+ and MPI groups. In addition, serum estradiol was increased in the SPI− group. These results are similar to a study in young men by Dillingham et al. (20) in which a low-isoflavone protein isolate containing <2 mg/d isoflavones significantly increased estradiol and estrone compared with a milk protein isolate after a 8-wk intervention. Our results differ, however, from a study in older men by Goldin et al. (19) in which a low-isoflavone soy protein isolate containing <2 mg/d isoflavones did not change estradiol or estrone concentrations after a 6-wk intervention. Interestingly, we found serum estradiol was significantly higher in the SPI− group than in the SPI+ group, whereas in Dillingham et al. (20) found that estradiol in the low-isoflavone group did not differ from the high-isoflavone group (20).

Serum androstenedione and DHEAS concentrations were increased in the SPI− group compared with both SPI+ and MPI groups. No other soy protein or isoflavone intervention study has

reported a change in circulating androstenedione (12,17,19,20,30), but all other studies to date have intervened for a shorter duration. Higher DHEAS is consistent with other low-isoflavone soy protein isolate interventions (19,20). Although DHEAS and androstenedione can be converted by 17β-hydroxysteroid dehydrogenase to testosterone, no significant changes were observed in circulating testosterone, free testosterone, or DHT. Instead, our study population had low, but normal, testosterone concentrations throughout the study. Although DHEAS and androstenedione concentrations have been associated with aggressive prostate cancer (46), our findings of unchanged testosterone and a trend toward lower AR expression ($P = 0.09$) suggest neutral effects of SPI− consumption. In fact, because DHEAS and androstenedione may be converted to estradiol and estrone in the prostate gland (47), the increase in DHEAS and androstenedione may have contributed to the observed increases in circulating estradiol and estrone. The hormonal effects of SPI− consumption are likely due to the effects of the alcohol extraction process on SPI constituents.

In conclusion, we found that consumption of isoflavone-rich soy protein for 6 mo lowered AR expression levels in the prostate, but did not change ERβ expression or circulating hormones in men at high risk of prostate cancer. Although consumption of the alcohol-extracted soy protein did not significantly lower AR expression, its effect appeared to be intermediate to that of SPI+ and MPI consumption, suggesting that the isoflavones alone may not be responsible for the AR expression decrease, or, alternatively, that the low level of isoflavones in SPI− were sufficient to alter the AR. Unexpectedly, consumption of SPI−, but not SPI+, significantly increased estradiol and androstenedione concentrations. None of these results were influenced by equol excretion status. These data suggest that consumption of isoflavone-rich and isoflavone-poor soy protein isolate exert differing effects on endogenous hormones and receptor expression, which may mediate prostate cancer preventive effects.

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Soy Protein Isolate Increases Urinary Estrogens and the Ratio of 2:16α-Hydroxyestrone in Men at High Risk of Prostate Cancer[1,2]

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Abstract

Specific estrogen metabolites may initiate and promote hormone-related cancers. In epidemiological studies, significantly lower excretion of urinary estradiol (E2) and lower ratio of urinary 2-hydroxy estrogens to 16α-hydroxyestrone (2:16 OH-E1) have been reported in prostate cancer cases compared to controls. Although soy supplementation has been shown to increase the ratio 2:16 OH-E1 in women, no studies to our knowledge have investigated the effects of soy supplementation on estrogen metabolism in men. The objective of this randomized controlled trial was to determine the effects of soy protein isolate consumption on estrogen metabolism in men at high risk for developing advanced prostate cancer. Fifty-eight men supplemented their habitual diets with 1 of 3 protein isolates: *1*) isoflavone-rich soy protein isolate (SPI+) (107 mg isoflavones/d); *2*) alcohol-washed soy protein isolate (SPI−) (<6 mg isoflavones/d); or *3*) milk protein isolate (MPI), each providing 40 g protein/d. At 0, 3, and 6 mo of supplementation, the urinary estrogen metabolite profile was measured by GC-MS. Both soy groups had higher E2 excretion than the MPI group at 3 and 6 mo. After 6 mo of supplementation, the SPI+ group had a significantly higher urinary 2:16 OH-E1 ratio than the MPI group. Increased urinary E2 excretion and 2:16 OH-E1 ratio in men consuming soy protein isolate are consistent with studies in postmenopausal women and suggest that soy consumption may be beneficial in men at high risk of progressing to advanced prostate cancer as a result of effects on endogenous estrogen metabolism. J. Nutr. 137: 2258–2263, 2007.

Introduction

Prostate cancer development is associated with andropause, when the ratio of circulating estrogens to androgens may increase by up to 40% (1). Increased estrogens are known to suppress testosterone production and compete with androgens for the androgen receptor. It has also been hypothesized that rising estrogen concentrations may cause direct mutagenic effects and unscheduled proliferation, in part due to the metabolism of the endogenous estrogens, estrone (E1)[6] and estradiol (E2) by cytochrome P450 (CYP) enzymes, with subsequent creation of more potent estrogens and electrophilic intermediates. It has been suggested that the 2-hydroxy estrogens are benign, the 2-methoxy estrogens may be anti-carcinogenic through detoxification of electrophilic intermediates, and the 4- and 16α-hydroxy estrogens may be carcinogenic (2,3).

Estrogen metabolism is regulated by the amount of substrate available and the expression and activity of CYP enzymes. In phase I metabolism, E1 and E2 are converted by CYP 1A/1B/3A to the relatively inactive metabolites 2-hydroxyestrone (2-OH-E1) and 2-hydroxyestradiol (2-OH-E2), respectively (4–6). Alternatively, E1 and E2 may be metabolized by CYP 1A/3A to 4-hydroxyestrone (4-OH-E1) and 4-hydroxyestradiol (4-OH-E2) (6,7), metabolites shown to initiate cancer in rats by forming mutagenic DNA adducts (2). E1 may also be metabolized to 16-α-hydroxyestrone (16α-OH-E1), a metabolite shown to covalently bind the estrogen receptor, signaling sustained estrogen receptor-mediated proliferation that may promote tumor growth (3,8). In phase II metabolism, most of the 2-hydroxy metabolites are conjugated by catechol-O-methyltransferase to 2-methoxyestradiol (2-ME2), a metabolite shown to inhibit carcinogenesis by inducing apoptosis and suppressing proliferation (9).

Most of the interest in estrogen metabolism and cancer has been in relation to breast cancer risk. Numerous studies have shown an inverse relationship between the ratio of urinary 2-hydroxy

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[6] Abbreviations used: CYP, cytochrome P450 enzymes; E1, estrone; E2, estradiol; 2-ME2, 2-methoxyestradiol; MPI, milk protein isolate; 2-OH-E1, 2-hydroxyestrone; 2:16 OH-E1 ratio, [(2-OH-E1 + 2-OH-E2)/16α-OH-E1]; 2-OH-E2, 2-hydroxyestradiol; 4-OH-E1, 4-hydroxyestrone; 4-OH-E2, 4-hydroxyestradiol; 16α-OH-E1, 16α-hydroxyestrone; SPI+, isoflavone-rich soy protein isolate; SPI−, alcohol-extracted soy protein isolate.

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estrogens to 16α-hydroxyestrone (2:16 OH-E1) and breast cancer risk (10–17), although a few studies have not shown a significant association (18–20) and 1 study found an association in premenopausal but not postmenopausal women (21). Although only 1 prostate cancer case-control study has been reported in men, results were similar, with a trend toward lower 16α-OH-E1 excretion, significantly higher 2-OH-E1 excretion, and a significantly higher 2:16 OH-E1 ratio in controls than cases (22). These data are consistent with a pilot study that reported an inverse relationship between 2-OH-E1 excretion and serum prostate specific antigen, a marker of prostate cancer (23).

In epidemiological studies, soy intake has been associated with decreased prostate cancer risk (24), but the mechanism is unknown and no studies have reported the effects of soy supplementation on urinary estrogen metabolism in men. In women, soy consumption has been shown to increase 2-OH-E1 excretion (25–28), decrease 16α-OH-E1 excretion (29), and increase the urinary 2:16 OH-E1 ratio (25,26,28,29). One study reported an increased urinary 2:16 OH-E1 ratio only in women who metabolized the soy isoflavone daidzein to equol (28).

The aim of this study was to assess the effects of 6-mo soy protein isolate consumption on urinary estrogen metabolites in men at high risk of prostate cancer. The effects of an isoflavone-rich soy protein isolate (SPI+) were compared to those of an isoflavone-poor soy protein isolate (SPI−) to elucidate whether isoflavones are the soy components responsible for altered estrogen metabolism. The underlying hypothesis was that SPI+ consumption would increase urinary E2 and E1, 2-OH-E1, 2-ME2, and the 2:16 OH-E1 ratio, and decrease-16α-OH-E1, 4-OH-E1, and 4-OH-E2 excretion.

Materials and Methods

The study population, design, and treatment used in this Soy and Prostate Cancer Prevention trial have been discussed previously in detail (30). All 58 participants were recruited by urologic physicians at the Minneapolis Veteran's Administration Medical Center. The subjects were men between the ages of 50 and 85 y who recently underwent a prostate biopsy. Trained pathologists evaluated biopsy cores through routine histology diagnosis. Men were excluded from the trial if they were morbidly obese (BMI > 40 kg/m^2), had prostate cancer that required medical treatment, had chronic prostatitis, consumed more than 14 alcoholic drinks per week, were allergic to soy or milk, used antibiotics frequently, or were on medically prescribed protein-restricted diets. All subjects provided written informed consent for participation in the trial, which was approved by the University of Minnesota Institutional Review Board: Human Subjects Committee, the Minneapolis Veterans Affairs Institutional Review Board and the U.S. Army Medical Research and Materiel Command's Human Subjects Research Review Board.

The subjects were randomly assigned to consume 1 of 3 protein isolates for 6 mo: *1*) SPI+ containing 107 ± 5.0 mg isoflavones/d expressed as aglycone equivalents; *2*) alcohol-extracted SPI− containing <6 ± 0.7 mg isoflavones/d expressed as aglycone equivalents; or *3*) milk protein isolate (MPI) containing 0 mg isoflavones/d (Solae Company). The protein isolates were taken in divided doses twice daily, contributing a total of 40 g of protein and 200–400 kcal (1 kcal = 4.184 kJ) to the subjects' habitual diets each day. The mean distribution of isoflavones was 53% genistein, 35% daidzein, and 11% glycitein in SPI+, and 57% genistein, 20% daidzein, and 23% glycitein in SPI−, as analyzed by HPLC in the laboratory of Dr. Pat Murphy, Department of Food Science and Human Nutrition, Iowa State University. Participants recorded the time of consumption in study calendars and compliance was assessed by self-report as detailed previously (30). To prevent any other soy isoflavone consumption, subjects were given a detailed list of soy-containing products to avoid.

The men collected 24-h urine samples 1 d prior to each of 3 clinic visits at 0, 3, and 6 mo. The urine was collected in opaque plastic containers containing 1 g ascorbic acid/L, then was preserved with 0.1% sodium azide, and aliquots were stored at –20°C until analysis. Urinary creatinine was measured by dry slide chemistry with a VITROS Clinical Chemistry analyzer (Ortho-Clinical Diagnostics) and equol concentration was determined by HPLC-MS as previously described (31). For equol concentrations, the intra-assay CV was 8.2% and the inter-assay CV was 12.5%.

Estrogen metabolites were measured by GC-MS using the method described below, modified from previously described methods (29,32). Urine samples were thawed at room temperature, thoroughly mixed by vortex to ensure homogeneity, and centrifuged at 5°C for 5 min. Duplicate 10-mL aliquots of urine were added to clean, silanized 30-mL screw-top test tubes. Deuterated standards (C/D/N Isotopes, Pointe-Claire) of all estrogen metabolites assayed were added to the urine and an equal volume (10 mL) of ethoximation solution was added to the test tubes, thoroughly mixed by vortex and inversion, and incubated overnight at room temperature (~20–25°C).

The following day, the ethoximated samples were applied to Bond Elute LRC C-18 columns (Varian; 500 mg/column). The C-18 columns had been preconditioned with 5 mL methanol and 10 mL of deionized-distilled water immediately prior to sample introduction. Columns were then washed with 5 mL of 0.15 mol/L acetate buffer, pH 3.0. Samples were eluted into a clean, silanized test tube with 3.0 mL of methanol and then evaporated to dryness under nitrogen. The dry samples were hydrolyzed by dissolving in 5 mL of a solution containing 25 mg ascorbic acid and 50 μL β-glucuronidase (Sigma no. G-7770, crude extract from *Helix pomatia*) in 0.15 mol/L acetate buffer, pH 4.1, and incubated overnight at 37°C.

The following day, the hydrolyzed samples were applied to C-18 columns (conditioned as above), washed with 5 mL of deionized-distilled water, and eluted into clean, silanized test tubes with 4.0 mL of methanol. Samples were evaporated to dryness under nitrogen and derivized to their trimethylsilyl components with 200 μL of a 15% MSTFA+ TMCS solution in acetonitrile (MSTFA+ 1% TMCS, Pierce Biotechnology, product no. 48915).

Chromatographic analysis was performed on an HP 5890 Series II gas chromatograph equipped with an HP-1MS 15-m column (0.25-mm i.d., 0.25-μm film thickness) interfaced to an HP 5970 mass selective detector. Instrumental programmed control and quantitative analysis was performed using HP Chemstation software. All samples from a given subject were analyzed in the same batch and an equal number of subjects from each group were included in each batch. Intra-assay CV were between 3.5 and 6.4% and inter-assay CV were between 4.3 and 13.0%. Detection limits were 1.0 μg/L for all estrogen metabolites except 2-OH-E2 and 2-OH-E1, which had detection limits of 0.50 μg/L. 4-OH-E2, 4-OH-E1, 4-methoxyestradiol, and 4-methoxyestrone were undetectable in all subjects.

Subject retention. Subject accrual has been described previously in detail (30), with some variation described below. One subject who consented to the Soy and Prostate Cancer Prevention trial refused to collect his urine and 2 other subjects were excluded from analysis due to missing baseline urine collections. Four subjects did not collect urine at all 3 time points [no mid-point urine ($n = 1$), no final urine ($n = 3$)]. Thus, 55 participants were evaluated at baseline, 54 were evaluated at 3 mo, and 52 were evaluated at 6 mo. One subject did not consume the treatment powder for 3 d prior to his 6-mo appointment as a result of illness, so his data were excluded from the 6-mo equol excretion analysis.

Statistical analysis. Demographic comparisons between groups were performed with 1-way ANOVA for continuous endpoints and chi-square for categories of prostate cancer markers. ANCOVA (SAS Proc GLM) was used to compare group means adjusted by their baseline values (33). For 16α-OH-E1, the model included a bodyweight by baseline metabolite interaction. In addition, preplanned pairwise comparisons as dictated by the study hypotheses were carried out. Paired *t* tests were used to test for significant within-group changes. Skewed data were log transformed before analysis and results are reported as geometric means and 95% CI. Data were analyzed both as nanomoles per day and nanomoles per milligram creatinine, and because there were no differences, data are

expressed as nanomoles per day. Statistical significance was defined as $P < 0.05$.

Results

Baseline. All 3 groups (SPI+, SPI−, and MPI) had similar anthropometrics, cancer status, and dietary intake (**Table 1**). The mean age of the men was 68 y, and mean BMI was 30 kg/m^2. The only significant difference among groups was that the SPI− group consumed more protein at baseline, but not at 3 and 6 mo (30). Baseline urinary estrogen metabolites were similar except that the SPI+ group had higher 2-ME2 than the MPI group (**Table 2**).

Urinary estrogen metabolites. In both soy groups, E2 concentrations significantly increased from baseline at 3 and 6 mo and were significantly higher than the MPI group at both time points (Table 2). A similar pattern was seen with respect to E1 concentrations at 6 mo. 2-OH-E1 concentrations did not differ over 6 mo, but at 6 mo, 2-OH-E2 concentration decreased significantly from baseline in the MPI group and was significantly lower than both soy groups. Both soy groups showed higher 16α-OH-E1 concentrations than the MPI group at 3 mo, but this disappeared at 6 mo. The 2:16 OH-E1 ratio was significantly higher in the SPI+ group than the MPI group at 6 mo.

Equol-excretor status. Equol excretor status was determined only in the SPI+ group, which received sufficient daidzein for equol production. There were 4 excretors and 15 nonexcretors. However, only 1 excretor remained at 6 mo, because 1 dropped out of the study after 3 mo, data were excluded from another subject as discussed above, and 1 apparently changed excretor status. Therefore, only the 3-mo data are reported. Baseline anthropometrics, cancer status, and dietary intake between excretors and nonexcretors did not differ (30). At baseline, the equol excretors tended to have higher 2:16 OH-E1 concentrations than nonexcretors ($P = 0.08$) (**Table 3**). All measured estrogen metabolites were the same between equol excretors and nonexcretors after 3 mo of SPI+ consumption.

TABLE 1 Baseline characteristics of subjects[1]

	SPI+, $n = 19$	SPI−, $n = 19$	MPI, $n = 17$
Age, *y*	68 ± 8	68 ± 6	69 ± 6
Body weight, *kg*	91 ± 16^{ab}	89 ± 12^{a}	98 ± 15^{b}
Height, *cm*	174 ± 7	174 ± 8	177 ± 8
BMI, *kg/m^2*	30 ± 5	30 ± 4	32 ± 6
Prostate cancer markers,[2] *n* (%)			
Prostatic intraepithelial neoplasia	14 (74)	12 (63)	13 (76)
Atypical small acini suspicious for prostatic adenocarcinoma	3 (16)	6 (32)	2 (12)
Prostate cancer	2 (11)	1 (5)	2 (12)
Dietary intake			
Energy intake, *kcal/d*[3]	2088 ± 590	2335 ± 590	2092 ± 530
Protein, *g/d*	80 ± 15^{a}	103 ± 24^{b}	81 ± 25^{a}
Carbohydrate, *g/d*	254 ± 109	270 ± 116	239 ± 59
Fat, *g/d*	83 ± 31	96 ± 29	89 ± 24
Dietary fiber, *g/d*	17 ± 9	18 ± 7	17 ± 5

[1] All values are means ± SD except prostate cancer markers, which are *n* (%). Means in a row with superscripts without a common letter differ, $P < 0.05$.
[2] Subjects were categorized by most advanced prostate cancer marker.
[3] 1 kcal = 4.184 kJ.

TABLE 2 Urinary estrogen metabolites in men at high risk of prostate cancer that consumed various protein isolates for 6 mo[1]

	SPI+, $n = 19$[2]	SPI−, $n = 19$[2]	MPI, $n = 17$[2]
E2		*nmol/d*	
Baseline	51 (34, 74)	42 (27, 63)	49 (29, 81)
3 mo	94 (65, 135)^{a}*	76 (53, 111)^{a}*	44 (30, 66)^{b}
6 mo	91 (63, 132)^{a}*	90 (63, 129)^{a}*	50 (34, 72)^{b}
E1			
Baseline	20 (15,28)	18 (13,26)	25 (20,31)
3 mo	26 (20,34)	21 (16,28)	22 (16,29)
6 mo	37 (28, 49)^{a}*	27 (20,35)^{b}*	23 (17,30)^{b}
2-ME2			
Baseline	57 (39, 84)^{a}	42 (31, 56)^{ab}	31 (21, 47)^{b}
3 mo	39 (26, 58)	42 (28, 62)	37 (24, 57)
6 mo	26 (18,38)	36 (25, 52)	34 (23, 50)
2-ME1			
Baseline	9.1 (7,12)	9.5 (6,15)	8.1 (6,12)
3 mo	7.6 (5,12)	9.5 (6,15)	8.9 (6,14)
6 mo	9.8 (7,15)	9.2 (6,14)	7.9 (5,12)
E3			
Baseline	55 (42, 71)	28 (14, 57)	47 (29, 78)
3 mo	28 (19,42)^{a}	56 (38, 84)^{b}	41 (27, 63)^{ab}
6 mo	31 (19, 49)	45 (28, 72)	42 (26, 67)
2-OH-E2			
Baseline	7.3 (5,12)	4.8 (3,8)	5.9 (3,11)
3 mo	7.2 (5,11)	6.9 (4,11)	5.6 (4,9)
6 mo	5.6 (4,8)^{a}	8.3 (6,12)^{a}	3.0 (2,4)^{b}*
2-OH-E1			
Baseline	20 (13,30)	21 (13,33)	26 (19,36)
3 mo	21 (14,30)	23 (16,34)	26 (17,38)
6 mo	29 (21,41)	25 (18,35)	21 (15,30)
16α-OH-E1			
Baseline	6.0 (4,9)	5.7 (4,9)	6.9 (5,10)
3 mo	7.4 (5,11)^{a}	7.9 (5,11)^{a}	4.5 (3,7)^{b}
6 mo	6.0 (4,9)	7.3 (5,11)	6.6 (5,10)
2:16 OH-E1 ratio (mean ± SE)			
Baseline	7.8 ± 1	7.5 ± 1	7.8 ± 2
3 mo	8.0 ± 2	8.5 ± 2	10.4 ± 2
6 mo	11.3 ± 2^{a}	8.2 ± 2^{ab}	5.1 ± 2^{b}

[1] Baseline data are unadjusted geometric means (95% CI) except 2:16 OH-E1 ratio data, which are means ± SE. All other data are least-squares geometric means adjusted for baseline measurement ± 95% CI, except 16α-OH-E1, which is additionally adjusted for baseline weight, and 2:16 OH-E1 ratio data, which are least-squares means ± SE and were analyzed on the original scale. Means in a row with superscripts without a common letter differ, $P < 0.05$. *Different from baseline, $P < 0.05$.
[2] Sample sizes listed at column headings are for all time points except the 3-mo MPI, $n = 16$; 6-mo SPI+, $n = 17$; and 6-mo SPI−, $n = 18$ time points.

Discussion

The primary objective of this study was to evaluate the effects of soy protein isolate consumption on the urinary estrogen profile in men at risk for developing advanced prostate cancer. Consumption of soy protein isolate, regardless of isoflavone content, increased urinary excretion of E2 and tended to increase excretion of E1. These results are similar to those from studies in postmenopausal women (27) and may be clinically relevant to prostate cancer prevention. Higher urinary excretion of E2 has been observed in prostate cancer controls compared to cases (34) and a high E2 concentration in the blood has been associated

TABLE 3 Urinary estrogen metabolites between equol excretors and nonexcretors in men at high risk of prostate cancer who consumed SPI+ for 6 mo[1]

	Excretors, $n = 4$	Nonexcretors, $n = 15$
E2	*nmol/d*	
Baseline	49 (21, 115)	51 (32, 82)
3 mo	76 (26, 224)	105 (60, 183)
E1		
Baseline	25 (16,37)	19 (13,28)
3 mo	23 (11, 49)	26 (18,38)
2-ME2		
Baseline	56 (16, 201)	58 (37, 91)
3 mo	29 (10, 82)	46 (27, 79)
2-ME1		
Baseline	9.0 (4,20)	9.1 (6,13)
3 mo	11 (5,25)	6.9 (5,11)
E3		
Baseline	76 (35, 167)	50 (37, 67)
3 mo	20 (5, 81)	37 (19, 74)
2-OH-E2		
Baseline	10.7 (1, 98)	6.6 (4,11)
3 mo	3.4 (1,10)	9.6 (5,17)[2]
2-OH-E1		
Baseline	29 (6, 132)	18 (11,29)
3 mo	16 (5, 54)	21 (11,39)
16α-OH-E1		
Baseline	6.3 (4,23)	5.9 (4,9)
3 mo	12 (5,28)	6.1 (4,9)
2:16 OH-E1 ratio (means ± SE)		
Baseline	12.0 ± 2	6.4 ± 1
3 mo	8.0 ± 7	8.2 ± 1

[1] Baseline data are unadjusted means ± 95% CI except 2:16 OH-E1 ratios, which are means ± SE. Data from 3 mo are least-squares means adjusted for baseline measurement ± SE, except 16α-OH-E1, which is additionally adjusted for interaction between treatment and baseline, and 2:16 OH-E1 ratio data which are least-squares means ± SE and were analyzed on the original scale.
[2] $n = 13$.

with decreased prostate cancer risk (35–37). Estrogens or estrogen analogs have been prescribed for decades to prostate cancer patients in order to decrease androgen production through negative feedback on the hypothalamic-pituitary-gonadal system. Despite our previous finding that soy consumption does not alter circulating estrogen concentrations (30), our observations of increased E2 excretion support the hypothesis that effects on estrogens may be 1 mechanism by which soy supplementation reduces the risk of prostate cancer. Our observation that this effect occurred regardless of isoflavone content of the soy protein isolate suggests that isoflavones may not be the only hormone-modulating compound in soy. Alternatively, it is possible, although unlikely, that the small dose of isoflavones in the SPI− was sufficient to elicit a hormonal response.

We previously showed consumption of SPI−, but not SPI+, increased serum E2 and E1 concentrations in this population (30). Given that our subjects did not have kidney disease and that hormone concentrations fluctuate throughout the day, it is possible that our 24-h urine data, analyzed by GC-MS, better reflect total estrogen exposure than circulating concentrations determined by 1 blood draw analyzed by radioimmunoassay. This speculation is based on a chapter written by Adlercreutz (38) suggesting that the GC-MS analytical method used for urinary estrogen metabolites is more accurate than the radioimmunoassay method used for serum hormone analysis and that a longer time frame captured in the biological sample (24 h) may be more indicative of hormone exposure than a single blood draw. Conversely, increased urinary E2 excretion over time could decrease systemic E2 exposure; however, we did not find that serum E2 concentrations were inversely correlated to urinary E2 in our 6-mo study.

Urinary 2-OH-E2 excretion decreased in the MPI group but not in the soy groups, possibly due to higher E2 concentrations in the soy groups providing more substrate for the 2-hydroxy pathway than the control group. It has been suggested that soy consumption alters the enzymes involved in the formation of 2-hydroxy metabolites, including CYP 1A/3A (25,39), although the data are somewhat inconsistent (39,40).

Both soy groups had significantly higher urinary 16α-OH-E1 excretion than the MPI group at 3 mo. These results are consistent with 1 study in postmenopausal women in which consumption of soy protein isolate containing 44 mg isoflavones/d tended to increase urinary excretion of 16α-OH-E1 after a 6-wk intervention (28). On the other hand, postmenopausal women who consumed soy protein isolate containing 132 mg isoflavones/d for 3 mo (27) and premenopausal women who consumed soy protein isolate containing 129 mg isoflavones/d for 3 mo (29) both had decreased urinary 16α-OH-E1 excretion. Others have reported no effects of soy protein consumption on urinary 16α-OH-E1 excretion in women (25,41,42).

Most importantly, this is the first study, to our knowledge, to show that soy protein isolate consumption alters the urinary ratio of 2:16 OH-E1 in men. The 2:16 OH-E1 ratio was higher in the SPI+ group than in the MPI group at 6 mo, consistent with data from soy intervention studies performed in women (25,26,28,29). An increased 2:16 OH-E1 ratio has been associated with reduced risk of breast cancer in numerous studies (10–17,43), but only 1 study has been published evaluating the relationship between the 2:16 OH-E1 ratio and prostate cancer risk (22). This study showed that an increased 2:16 OH-E1 ratio was associated with reduced risk of prostate cancer (22). This finding suggests that 1 of the mechanisms by which consumption of SPI+ may prevent prostate cancer is via reducing the genotoxic effects of estrogen metabolites.

Within the SPI+ group, equol excretors tended to have a higher 2:16 OH-E1 ratio than nonexcretors at baseline. This finding is consistent with data suggesting that there may be beneficial differences between equol excretors and nonexcretors unrelated to the biological activity of equol itself (26,44,45). Our observation of no difference in the effects of soy consumption by equol excretor status was similar to previous reports in premenopausal and postmenopausal women (27,29), although a few studies in women have reported an association between urinary equol excretion and a higher 2:16 OH-E1 ratio (26,28,46). Our analysis was likely limited by the small sample size and the results are preliminary and should be interpreted with caution.

To our knowledge, this is the first study to report the full profile of urinary estrogen metabolites in men at high risk of developing prostate cancer and the first to report the effects of soy consumption on estrogen metabolite excretion in men. Consumption of soy protein isolate, regardless of isoflavone content, increased estrogen excretion, and SPI+ consumption but not SPI− increased the 2:16 OH-E1 ratio. Given that increased estrogens and 2:16 OH-E1 ratio have been associated with lower prostate cancer risk, our data suggest that effects on endogenous estrogen synthesis and metabolism may contribute to the prostate cancer preventive effects of soy consumption.

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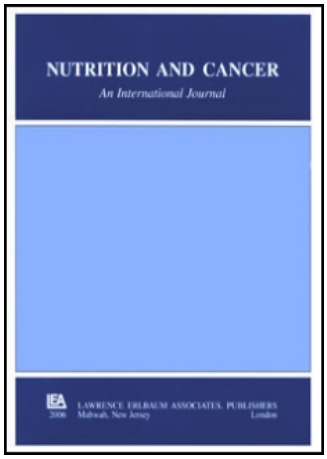

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Effects of Soy Protein Isolate Consumption on Prostate Cancer Biomarkers in Men With HGPIN, ASAP, and Low-Grade Prostate Cancer

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Fifty-eight men at high risk of prostate cancer or with low-grade prostate cancer were randomly assigned to consume 1 of 3 protein isolates containing 40 g protein: 1) soy protein (SPI+, 107 mg isoflavones/d); 2) alcohol-washed soy protein (SPI–, <6 mg isoflavones/d); or 3) milk protein (MPI). Proliferating cell nuclear antigen (PCNA), epidermal growth factor receptor, B-cell non-Hodgkin lymphoma-2 (Bcl-2), and Bcl-2-associated X protein (Bax) were assessed in baseline and ending prostate biopsy cores. Serum collected at 0, 3, and 6 mo was analyzed for total and free prostate specific antigen (PSA). Consumption of SPI+ did not alter any of the prostate cancer tumor markers. Bax expression decreased from baseline in the SPI– group, resulting in lower Bax expression than the MPI group. PCNA expression also decreased from baseline in the SPI– group, but this was not different from the other 2 groups. PSA did not differ among the groups at 3 or 6 mo. Interestingly, a lower rate of prostate cancer developed in the soy groups compared to the milk group ($P = 0.01$). These data suggest that 6-mo SPI+ consumption does not alter prostate tissue biomarkers, SPI– consumption exerts mixed effects, and less prostate cancer is detected after 6 mo of soy consumption regardless of isoflavone content.

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INTRODUCTION

Men with biopsy-proven, low-grade cancer, or preneoplastic lesions such as atypical small acini suspicious for prostatic adenocarcinoma (ASAP) or high-grade prostatic intraepithelial neoplasia (HGPIN) would be ideal candidates for a nontoxic dietary supplement with proven efficacy for reversing or retarding these early prostate tissue lesions. Phytoestrogenic soy isoflavones have been shown to exert prostate cancer preventive effects, and soy consumption has been associated with decreased prostate cancer risk in epidemiological studies (1). Isoflavone supplementation has been shown to suppress serum prostate specific antigen (PSA), a biomarker associated with prostate cancer progression. In men with prostate cancer, soy food interventions have significantly decreased mean total serum PSA compared to controls (2,3), although several studies have not shown statistically significant effects of soy or isoflavone consumption on total PSA (4–15). Because total PSA is a nonspecific biomarker for prostate cancer, clinicians often evaluate the free to total PSA percent to differentiate between cancer and benign conditions (16). The lower the value of free PSA percent, the greater the probability that elevated PSA represents cancer and not benign prostatic hyperplasia. In men with PSA concentrations between 4 and 10 ng/ml and a free PSA percent below 10%, risk of cancer is 56% compared to men with a free PSA percent above 25% whose risk of cancer is only 8% (17). Only two studies to date have evaluated the effects of soy or isoflavone consumption on free PSA percent. Dalais et al. (3) reported that soy grits increased free PSA percent, but Kranse et al. (10) did not observe a change in free PSA percent with isoflavone supplementation.

It has been suggested that intraprostatic expression of antigens related to carcinogenesis may be useful molecular biomarkers in dietary intervention studies (18). Soy isoflavone interventions in various models have decreased cell proliferation, downregulated the epidermal growth factor receptor (EGFr), and increased programmed cell death or apoptosis. Soy has suppressed cell proliferation as detected by proliferating cell nuclear antigen (PCNA) staining in rodents dosed with either soy protein concentrate (19) or physiologic concentrations of the isoflavone genistein (20). Physiological doses of dietary genistein have downregulated EGFr messenger RNA (mRNA) expression during the early phase of prostate cancer development in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model (20,21). Increased prostate tissue apoptosis has been shown in prostatectomy specimens obtained from patients treated with isoflavones derived from red clover when compared to historically matched controls (8), and soy protein concentrate has increased apoptotic index in the immune compromised mouse model (19). Genistein has increased apoptosis in vitro as detected by the proapoptotic signaling protein, B-cell non-Hodgkin lymphoma-2 (Bcl-2)-associated X protein (Bax) (22). Comparing Bax to the antiapoptotic signaling protein, Bcl-2 may indicate apoptosis status in prostate biopsy specimens (23,24).

The aim of this study was to assess the effects of soy protein isolate consumption on prostate cancer biomarkers in men at high risk of prostate cancer. A randomized placebo-controlled trial was performed in 58 men who consumed either isoflavone-rich soy protein isolate, isoflavone-poor soy protein isolate, or milk protein isolate for 6 mo. The purpose of this study was to evaluate the efficacy of a soy intervention at the beginning of prostate carcinogenesis and to determine whether isoflavones are the responsible bioactive components of soy. The underlying hypothesis was that isoflavone-rich soy protein isolate consumption would increase Bax and decrease Bcl-2, EGFr, PCNA, and serum PSA.

MATERIALS AND METHODS

The 6-mo randomized controlled trial was conducted at the Minneapolis Veteran's Administration Medical Center and was approved by the University of Minnesota Institutional Review Board: Human Subjects Committee, the Minneapolis Veterans Affairs Institutional Review Board, and the U.S. Army Medical Research and Materiel Command's Human Subjects Research Review Board. Subjects were recruited from a pool of patients who, due to their high-risk status, had already undergone a transrectal ultrasound and biopsy, and the biopsy results showed either preneoplastic lesions ($n = 53$) or low-grade prostate cancer with Gleason scores of 6 or below ($n = 5$). Subjects were considered high risk if they had high-grade prostatic intraepithelial neoplasia (PIN) ($n = 40$) or ASAP (atypical small acinar proliferation) ($n = 13$). The 5 patients with low-grade prostate cancer had elected to undergo active surveillance. Urologists invited patients to participate in the study at their postbiopsy clinic visit, and the patients' medical records were reviewed by a research nurse to determine eligibility. Patients were not allowed to participate if they were morbidly obese [body mass index (BMI) $>$ 40 kg/m^2], had prostate cancer that required medical treatment, had chronic prostatitis, consumed more than 14 alcoholic drinks per week, were allergic to soy or milk, used antibiotics frequently, or were on medically prescribed protein-restricted diets.

All 58 subjects supplemented their habitual diets twice daily with 1 of 3 study protein isolates: 1) soy protein isolate (SPI+); 2) alcohol-extracted soy protein isolate (SPI–); or 3) milk protein isolate (MPI; The Solae Company, St. Louis, MO). The protein isolates provided 40 g protein/day and 200–400 kcal/day. The isoflavone content (analyzed by Dr Pat Murphy, Department of Food Science and Human Nutrition, Iowa State University) was 107 $\pm$ 5.0 mg/day for the SPI+; $<$6 $\pm$ 0.7 mg/day for the SPI–; and 0 mg/day for the MPI (mean $\pm$ SD) expressed as aglycone equivalents. The mean distribution of isoflavones was 53% genistein, 35% daidzein, and 11% glycitein in SPI+ and 57% genistein, 20% daidzein, and 23% glycitein in SPI–. Compliance was assessed by self-report as detailed previously (25). To minimize isoflavone consumption from other sources, subjects were given a detailed list of soy-containing products to avoid.

Subject retention has been previously described in detail (25). Data from 58 participants were included in the serum PSA analysis ($n = 58$), and data from 44 subjects were included in the antigen expression analysis. Fewer participants were eligible for antigen expression analysis because 7 subjects did not undergo the final prostate biopsy [liver cancer ($n = 1$), heart complication ($n = 1$), not clinically indicated ($n = 1$), opted out of procedure ($n = 1$), and early withdrawal from study ($n = 3$)], and 7 subjects had insufficient biopsy tissue at either baseline or study end for the analyses.

Serum Collection and Analysis

Participants reported for clinic visits at 0, 3, and 6 mo. Fasting blood was drawn in the morning. Serum was separated and frozen at –70°C until analysis. Serum PSA was measured in one batch at the Minneapolis Veteran's Administration Hospital by the Architect total PSA chemiluminescence microparticle immunoassay (Architect ci8200, Abbott Laboratories, Chicago, IL). Intraassay variability was 2.5%. Free PSA was measured in one batch at Associated Regional and University Pathologists Laboratories by the Roche Modular E170 free PSA electrochemiluminescent immunoassay. Intraassay variability was 7.1%.

Tissue Collection and Analysis

Prostate cores were obtained before the initial screening and obtained again after the 6-mo dietary intervention. Biopsy cores were fixed in formalin and paraffin embedded. The paraffin-embedded blocks were sectioned onto slides, and the slides were

evaluated and diagnosed by the pathologist at the Minneapolis Veteran's Administration Hospital. After diagnosis, slides were obtained from pathology to perform immunohistochemistry for PCNA, EGFr, Bax, and Bcl-2 expression. The tissue sections were deparaffinized, rehydrated in graded alcohol, and transferred to phosphate-buffered solution (pH 7.3). Epitope retrieval was induced by pressure cooking at 103 kPa in citrate buffer with a pH of 6.0 for 10 min and submerged in quenching solution (3% H_2O_2 in 100% MeOH) for 5 min. After blocking (10% milk, 5% serum, and 1% bovine serum albumin), the samples were incubated overnight at 4°C with mouse monoclonal anti-PCNA antibody (555566; BD Biosciences, San Diego, CA; 1:500), mouse monoclonal anti-Bcl-2 antibody (551107; BD Biosciences, San Diego, CA; 1:500), or rabbit polyclonal anti-Bax antibody (554104; BD Biosciences, San Diego, CA; 1:1000). The samples for the EGFr assay were incubated at room temperature for 30 min with the mouse monoclonal anti-EGFr antibody (08-1205; Zymed, Invitrogen Corporation, Carlsbad, CA; ready to use). After rinsing, the samples were incubated with the corresponding biotinylated secondary antibody. Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) and diaminobenzidine (DAB) were utilized to stain the expressed antigens brown. The slides were rinsed with water and counterstained with Harris' Modified Hematoxylin (Fisher Scientific, Pittsburgh, PA). The slides were photographed, and the digital images were optimized for scoring as described in our previous report (25). Positive and negative controls were run in each batch, and the images were scored using the immunohistochemical histological score (HSCORE) semiquantative method (26). The HSCORE is a sum of the percentage of counted epithelial cells weighted by their staining intensity above control. HSCORE = Σ percentage of cells (PC) (i + 1); where i is the intensity of staining with a value of 0 (absent), 1 (weak), 2 (moderate), or 3 (strong) and PC is the percentage of stained epithelial cells for each intensity varying from 0–100%. The range of the HSCORE is a minimum of 1 and a maximum of 4. The vast majority of the glands scored represented normal, hyperplasic, or premalignant acini. The immunostained slides were evaluated independently by 2 technicians blinded to each patient's medical history. There was good agreement between the 2 observers; the Spearman correlation between them was 0.8. On average, 5 intact glands (range = 3–8) were scored per subject slide. Due to poor staining or incomplete glands on tissue sections, some slides were not scored; thus, a few patients were excluded from analysis: PCNA ($n = 3$) and EGFr ($n = 2$).

Statistical Analysis

Baseline comparisons between groups were performed with 1-factor analysis of variance for continuous endpoints and χ^2 for categories of prostate cancer markers. Analysis of covariance was used to compare groups adjusted for the baseline value of the final endpoint. Overall F tests comparing groups are reported, with preplanned pairwise comparisons of all groups for each endpoint as dictated by the study hypotheses. Paired t-tests were used to examine within-group changes. Prostate cancer incidence rates were compared between groups using Fisher's exact test and logistic regression. Statistical significance was defined as $P < 0.05$. All analyses were performed using SAS version 9.1 (27).

TABLE 1
Baseline pathological diagnosis[a]

Diagnosis	SPI+ ($n = 20$)	SPI– ($n = 20$)	MPI ($n = 18$)
PIN (n (%))	15 (75)	12 (60)	13 (72)
ASAP (n (%))	3 (15)	7 (35)	3 (17)
CaP (n (%))	2 (10)	1 (5)	2 (11)

[a] All values are n (%). Abbreviations are as follows: SPI+, isoflavone-rich soy protein isolate (40 g soy protein, 107 mg isoflavones/day); SPI–, alcohol-extracted soy protein isolate (40 g soy protein, <6 mg isoflavones/day); MPI, milk protein isolate (40 g milk protein); PIN, prostatic intraepithelial neoplasia; ASAP, atypical small acini suspicious for prostatic adenocarcinoma; CaP, prostate cancer. Subjects were categorized by most advanced pathological diagnosis.

RESULTS

Baseline

Anthropometrics and dietary intake did not differ between treatment groups as described previously (25). The average age for all men was 68 yr; the average BMI was 30 kg/m^2. At baseline, cancer status and aggregate antigen expression HSCORES did not differ among the groups (Tables 1 and 2). Similarly, there were no differences in baseline total or free PSA concentrations, prostate volume, or PSA density among the groups (Tables 3 and 4).

Antigen Expression

After 6 mo, Bax expression was lower in prostate biopsies in the SPI– group compared to the MPI group (pairwise comparison, $P = 0.03$) and approached a significant difference compared to the SPI+ group (pairwise comparison, $P = 0.10$; Table 2 and Fig. 1). PCNA expression was decreased from baseline in the SPI– group, but baseline-adjusted PCNA expression was not significantly different from the other two groups (Table 2 and Fig. 1). There were no effects of treatment or differences among the groups in Bcl-2, EGFr, Bax:Bcl-2 ratio, or Bax:PCNA ratio.

PSA and Prostate Volume

There were no effects of treatment or differences among the groups in total PSA, free PSA, or PSA percent (Table 3). Prostate volume at 6 mo was increased in the SPI– group relative to the MPI group (pairwise comparison, $P = 0.04$), but PSA density (serum total PSA/prostate volume) was not different among the groups (Table 4).

TABLE 2
Antigen expression (HSCORE) in benign prostate tissue[a]

	SPI+	SPI–	MPI
Bax[b]			
Baseline	1.38 ± 0.08	1.45 ± 0.07	1.35 ± 0.06
6 mo	1.41 ± 0.06[ab]	1.27 ± 0.05[a*]	1.44 ± 0.06[b]
PCNA[c]			
Baseline	1.61 ± 0.1	1.93 ± 0.1	1.86 ± 0.1
6 mo	1.69 ± 0.1	1.57 ± 0.1*	1.81 ± 0.1
Bcl-2[d]			
Baseline	1.11 ± 0.03	1.17 ± 0.07	1.09 ± 0.03
6 mo	1.15 ± 0.04	1.15 ± 0.04	1.19 ± 0.04
EGFr[e]			
Baseline	1.34 ± 0.08	1.42 ± 0.10	1.39 ± 0.11
6 mo	1.36 ± 0.06	1.37 ± 0.06	1.33 ± 0.06
Bax:Bcl-2 ratio[f]			
Baseline	1.25 ± 0.07	1.30 ± 0.10	1.23 ± 0.06
6 mo	1.20 ± 0.05	1.14 ± 0.05	1.22 ± 0.05
Bax: PCNA ratio[g]			
Baseline	0.88 ± 0.05	0.76 ± 0.05	0.76 ± 0.05
6 mo	0.89 ± 0.05	0.82 ± 0.05	0.84 ± 0.05

[a]Baseline data are unadjusted means + SE. All other data are least-squares means adjusted for baseline measurement ± SE. Abbreviations are as follows: HSCORE, immunohistochemical histological score; SPI+, isoflavone-rich soy protein isolate (40 g soy protein, 107 mg isoflavones/day); SPI–, alcohol-extracted soy protein isolate (40 g soy protein, <6 mg isoflavones/day); MPI, milk protein isolate (40 g milk protein); Bax, B-cell non-Hodgkin lymphoma-2 (Bcl-2)-associated X protein; PCNA, proliferating cell nuclear antigen; EGFr, epidermal growth factor receptor. Means in a row without a common subscript letter differ ($P < 0.05$). *, significant within-group change from baseline ($P < 0.05$).

[b]$n = 14$ for SPI+, n = 16 for SPI–, and $n = 14$ for MPI.

[c]$n = 14$ for SPI+, $n = 13$ for SPI–, and $n = 14$ for MPI.

[d]$n = 15$ for SPI+, $n = 14$ for SPI–, and $n = 16$ for MPI.

[e]$n = 15$ for SPI+, $n = 14$ for SPI–, and $n = 13$ for MPI.

[f]$n = 14$ for SPI+, $n = 14$ for SPI–, and $n = 13$ for MPI.

[g]$n = 13$ for SPI+, $n = 13$ for SPI–, and $n = 12$ for MPI.

TABLE 3
Serum PSA differences from baseline[a]

	SPI+ ($n = 20$)[b]	SPI– ($n = 20$)[b]	MPI ($n = 18$)[b]
Total PSA (ng/mL)			
Baseline	5.4 ± 1	5.0 ± 1	5.1 ± 1
3-mo change	−0.8 ± 0.5	−0.8 ± 0.5	−0.6 ± 0.6
6-mo change	−0.5 ± 0.6	−0.8 ± 0.6	−0.2 ± 0.6
Free PSA (ng/ml)			
Baseline	0.9 ± 0.09	0.8 ± 0.1	0.9 ± 0.2
3-mo change	−0.09 ± 0.09	0.04 ± 0.09	−0.10 ± 0.1
6-mo change	−0.07 ± 0.07	−0.02 ± 0.07	−0.06 ± 0.07
PSA %			
Baseline	22 ± 2	19 ± 2	22 ± 2
3-mo change	−0.21 ± 1	0.67 ± 1	−0.74 ± 1
6-mo change	1.03 ± 1	1.18 ± 1	−0.22 ± 1

[a]Baseline data are unadjusted means ± SE. Differences are post-intervention minus baseline and are least-squares means adjusted for baseline measurement + SE. Abbreviations are as follows: PSA, prostate specific antigen; PSA %, free PSA/total PSA; SPI+, isoflavone-rich soy protein isolate (40 g soy protein, 107 mg isoflavones/day); SPI–, alcohol-extracted soy protein isolate (40 g soy protein, <6 mg isoflavones/day); MPI = milk protein isolate (40 g milk protein).

[b]Sample sizes listed at column headings are for all time points except 3 mo MPI ($n = 17$), 6 mo SPI+ ($n = 18$), and 6 mo SPI– ($n = 19$).

Cancer Incidence

Of the 53 men without evidence of cancer at baseline biopsy, 49 completed the final prostate biopsy [elected not to undergo final biopsy procedure ($n = 2$), withdrew after 3 mo and elected not to follow up ($n = 1$), and advanced liver cancer ($n = 1$)]. Prostate cancer incidence was more than 6 times higher in the MPI group than in the combined soy groups ($P = 0.013$). Prostate cancer incidence was 38% ($n = 6/16$) in the MPI group vs. 6% ($n = 1/16$) in the SPI+ group and 6% ($n = 1/17$) in the SPI– group.

DISCUSSION

This study evaluated the effects of SPI on prostate tissue antigen expression levels, serum total and free PSA, prostate volume, and PSA density. Consumption of isoflavone-rich SPI had no effects on any of the prostate cancer tumor markers analyzed. However, in the postintervention biopsy tissue from the men consuming alcohol-extracted SPI, we observed lower Bax expression levels (reflecting decreased apoptosis) compared to those consuming MPI and decreased PCNA expression levels (reflecting decreased proliferation) compared to baseline values. Despite these seemingly contradictory effects, there was a trend toward decreased risk of cancer in the soy groups compared to the MPI group.

The lack of effect of SPI+ consumption on total PSA concentrations is consistent with several soy or isoflavone intervention studies in which no change in total PSA was observed (4–13) but inconsistent with a few reports of significant reductions (2) or trends toward reductions (14,15) in total PSA concentrations. Because a nearly significant difference in prostate volume was observed, we also evaluated total PSA standardized to prostate size (PSA density). Neither PSA density nor free PSA concentrations were different among the groups, which is consistent with all studies to date (3,4,10,12,14). We also found no effect of treatment on free PSA percent, which is consistent with Kranse et al. (10) but inconsistent with Dalais et al. (3), who reported increased free PSA percent. A limitation of this study was the lack of data available to calculate PSA doubling time or PSA velocity before and after the intervention.

TABLE 4
Prostate volume and PSA density differences from baseline[a]

	SPI+ ($n = 10$)	SPI– ($n = 13$)	MPI ($n = 15$)
Prostate volume (cm^3)			
Baseline	52 ± 5	47 ± 5	54 ± 6
6-mo change	-4.3 ± 3^{ab}	$1.6 \pm 2_a$	-5.5 ± 2^b
PSA density (ng/ml/cc)			
Baseline	0.1 ± 0.03	0.09 ± 0.02	0.1 ± 0.02
6-mo change	0.0001 ± 0.01	-0.003 ± 0.01	-0.005 ± 0.01

[a]Baseline data are unadjusted means ± SE. Differences are postintervention minus baseline and are least squares means adjusted for baseline measurement ± SE. Abbreviations are as follows: PSA, prostate specific antigen; PSA density, total PSA/prostate volume; SPI+, isoflavone-rich soy protein isolate (40 g soy protein, 107 mg isoflavones/day); SPI–, alcohol-extracted soy protein isolate (40 g soy protein, <6 mg isoflavones/day); MPI, milk protein isolate (40 g milk protein). Means in a row without a common subscript letter differ ($P < 0.05$).

Consumption of SPI+ did not affect the expression of the apoptotic cancer biomarkers, Bax and Bcl-2, analyzed in baseline and ending prostate biopsy cores. The lack of effect on apoptotic markers is in contrast to in vitro data showing increased Bax when LNCaP cells were exposed to 100 μM genistein (22), and one study that reported higher apoptotic index in prostate specimens obtained from men who consumed isoflavone extract compared to historically matched controls (8). The disparity between the results of this last study (8) and our results could be explained by different treatment regimens (red clover vs SPI), control groups (historically matched vs. placebo controlled), and analytical methods for apoptosis (apoptotic index vs specific signaling proteins, i.e., Bax and Bcl-2). Although comparing Bax to the antiapoptotic signaling protein Bcl-2 may indicate apoptosis status in prostate cancer biopsy specimens (23,24), most of our subjects did not have prostate cancer. Bcl-2 was scored only in the luminal layer, and consistent with the literature, we found that benign glands had minimal to absent staining of Bcl-2 in these cells (28). Given the small range in Bcl-2 HSCORE in our study, larger tissue sections and more subjects would need to be evaluated for improved reliability of the Bax to Bcl-2 ratio within precancerous lesions. Thus, utilizing biopsy cores from preneoplastic prostate glands for this endpoint was a limitation of our study design.

Although consumption of SPI+ did not influence Bax, consumption of SPI– significantly decreased Bax from baseline such that at 6 mo, it was lower than the other 2 groups. These findings are consistent with the hypothesis that isoflavones increase Bax and suggest that a different constituent of SPI+ decreases Bax, resulting in a neutral effect when they are present together and a reduction of Bax when isoflavones are removed (29–31). Given that this study is the first to evaluate these markers and reproducibility problems have been documented, this finding may also be explained by chance.

Consumption of SPI+ did not alter PCNA, whereas consumption of SPI– decreased PCNA from baseline, although there were no differences among the groups at 6 mo. These results are inconsistent with rodent studies that have shown that soy protein concentrate (19) or physiologic concentrations of genistein (20) suppressed PCNA staining. PCNA is an auxiliary protein of DNA polymerase that reaches maximal expression during the DNA replication phase (S phase) of the cell cycle. Therefore, abundant PCNA in the cell reflects DNA replication,

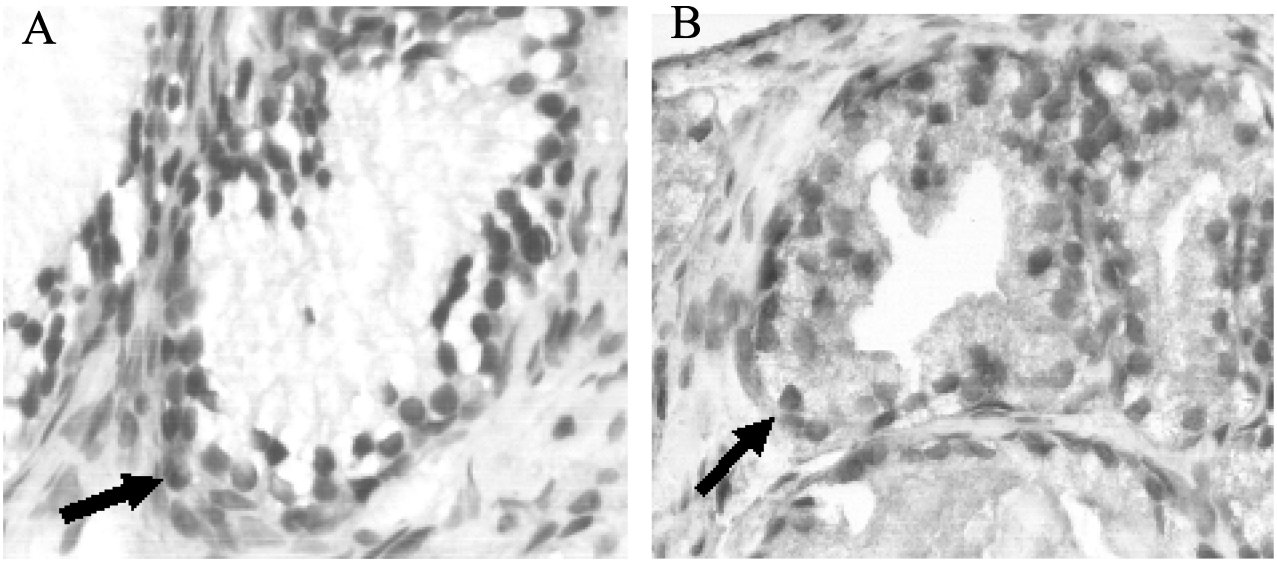

FIG. 1. Representative immunohistochemical staining of human prostate core biopsies for HSCORE (A) PCNA and (B) BAX. Arrow indicates stained acinar cell.

and several studies have confirmed that the PCNA index is directly correlated with prostate cancer progression (32–35).

We found no change in EGFr by either SPI+ or SPI– consumption, which is in contrast to animal studies in which dietary genistein downregulated EGFr mRNA expression during the early phase of prostate cancer development in the TRAMP model at physiologically plausible doses (20,21). EGFr activates transcription through either the EGFr-Shc-SOS-Ras-Raf-ERK1/2 or the phosphatidylinositol-3′kinase-AKT pathways leading to cellular proliferation, angiogenesis, and apoptosis evasion. A limitation of our study is that we measured EGFr expressed and not phosphorylated or activated. Thus, further studies are needed to evaluate the effects of SPI consumption on the activation of the EGFr pathways.

Last, we observed different rates at which patients progressed to a malignant diagnosis at study end. Malignancy was diagnosed 6 times more often in the MPI group than in the combined soy groups (SPI+ and SPI–). Though interesting and relevant, this finding should be interpreted with caution given that this short-term study was not designed to investigate progression to cancer. In light of data associating soy consumption with decreased prostate cancer risk in epidemiological studies (1) and mechanistic evidence of hormonal changes in this population (25,36), further soy interventions designed with prostate cancer onset or progression as endpoints are warranted.

To our knowledge, this is the first randomized controlled study on the effects of SPI intervention on prostate tissue biomarkers in men at high risk of developing prostate cancer. Consumption of isoflavone-rich SPI had no effects on any of the prostate cancer tumor markers analyzed. However, consumption of alcohol-washed (isoflavone-poor) SPI had mixed effects, decreasing proapoptotic Bax expression levels and decreasing proliferation as reflected in PCNA expression levels. These data suggest that there may be multiple constituents of SPI that exert varied effects on prostate cancer biomarkers. Importantly, we observed a lower rate of prostate cancer development in men in the soy groups compared to the milk group. Further research should be conducted to determine whether soy delays the onset and progression of clinically significant prostate cancer and to identify the responsible soy components.

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